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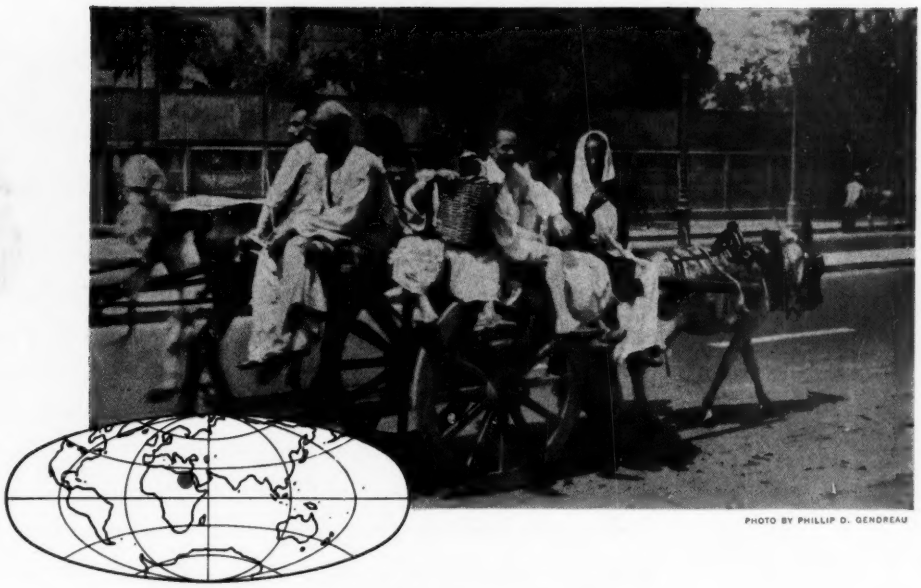

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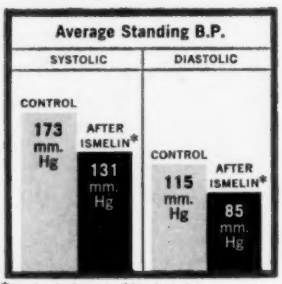
RESULTS: All 18 patients had reductions in standing blood pressure; 16 had moderate reductions in supine blood pressure as well. In 17 of the 18 cases, blood pressure levels became normal or near-normal in the erect position.



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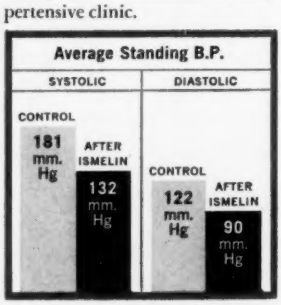
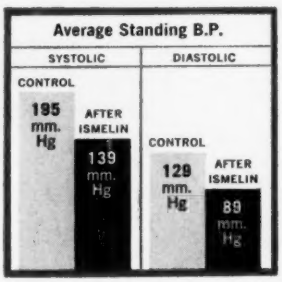
In 14 of 15 patients (93.3%) on Ismelin, blood pressure reduced to normal or near-normal levels in the standing position. Ismelin was administered orally by Frohlich and Freis[2] for 4 to 9 weeks to 15 male patients selected from the hypertensive clinic.



RESULTS: Ismelin evoked a potent antihypertensive response in the erect position: the blood pressure of 14 of the 15 patients dropped to normotensive or near-normotensive levels.

"The response [to Ismelin] was characterized by a potent, orthostatic, antihypertensive effect similar to that seen with the ganglionic blocking drugs but without the side-effects of parasympathetic blockade."[2]

In 15 of 18 subjects (83.3%), guanethidine [Ismelin] reduced high blood pressure to near-normotensive levels. Guanethidine [Ismelin] was administered orally by Richardson and Wyso[3] to 18 male hospitalized patients with hypertension.



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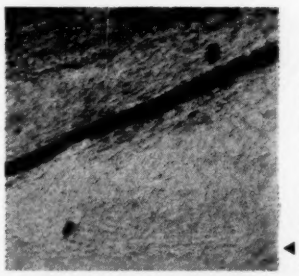
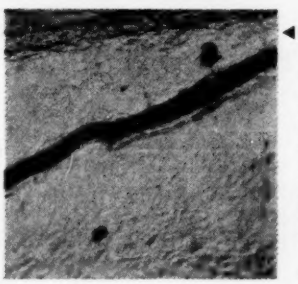
near-normal levels in 80 to 90% of cases[1-3]

RESULTS: "All patients showed definite reduction in blood pressure coincident with administration of [Ismelin]. In most of the subjects [15] standing blood pressure could be maintained near normal levels."[3]

"Side-effects encountered . . . have indeed been minimal . . ."[4] Brest and Moyer[4] state: "Side-effects [of Ismelin] encountered to date have indeed been minimal, with mild diarrhea as the only significant complaint even when large daily doses (450 mg.) of the drug are administered. No evidence of toxic action of the drug has been encountered thus far." Page[5] observes: "...Guanethidine [Ismelin] has the advantage [over ganglionic blockers] in that it is much easier to handle and does not produce nearly as much dose sensitivity. Too much of a ganglion-blocking agent will really 'clobber' the patient; with Guanethidine, there is much more leeway." Kirkendall and co-workers[6] report: "Guanethidine has remarkably few side effects. The absence of symptoms of parasympathetic blockade makes its use better tolerated by most patients than conventional ganglion blocking therapy." Leishman and associates[7] conclude: "The capacity of guanethidine to reduce the blood-pressure of hypertensive patients without symptoms of parasympathetic blockade is consistent with a mechanism of selective sympathetic-nerve inhibition..."





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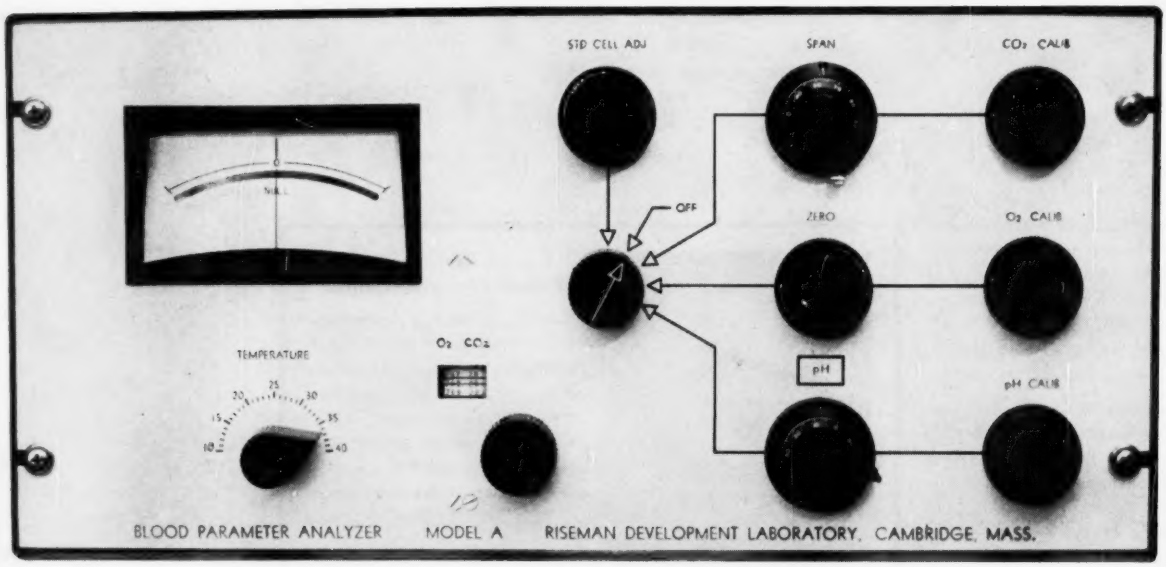

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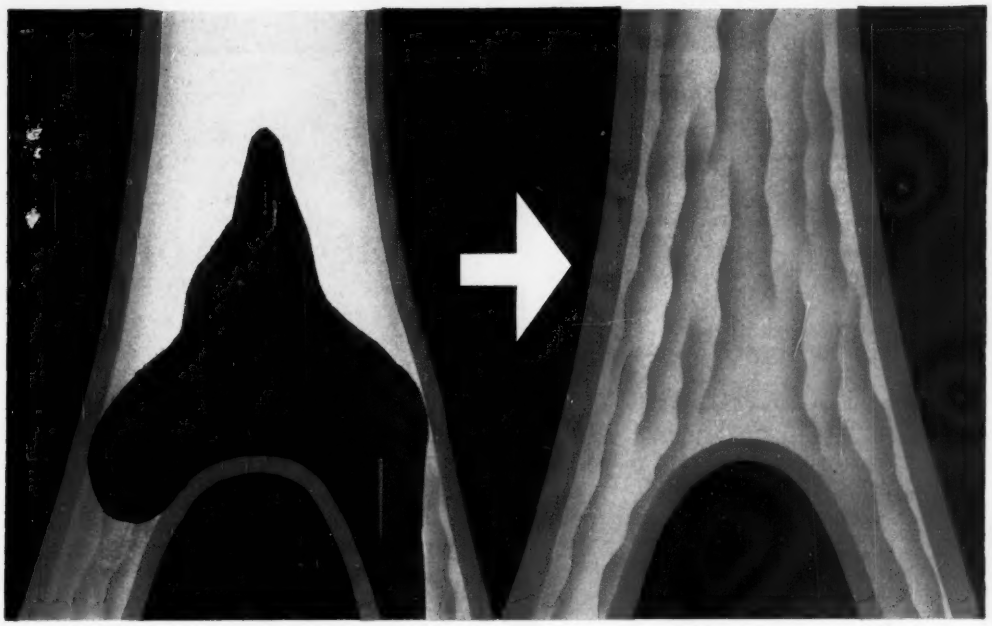

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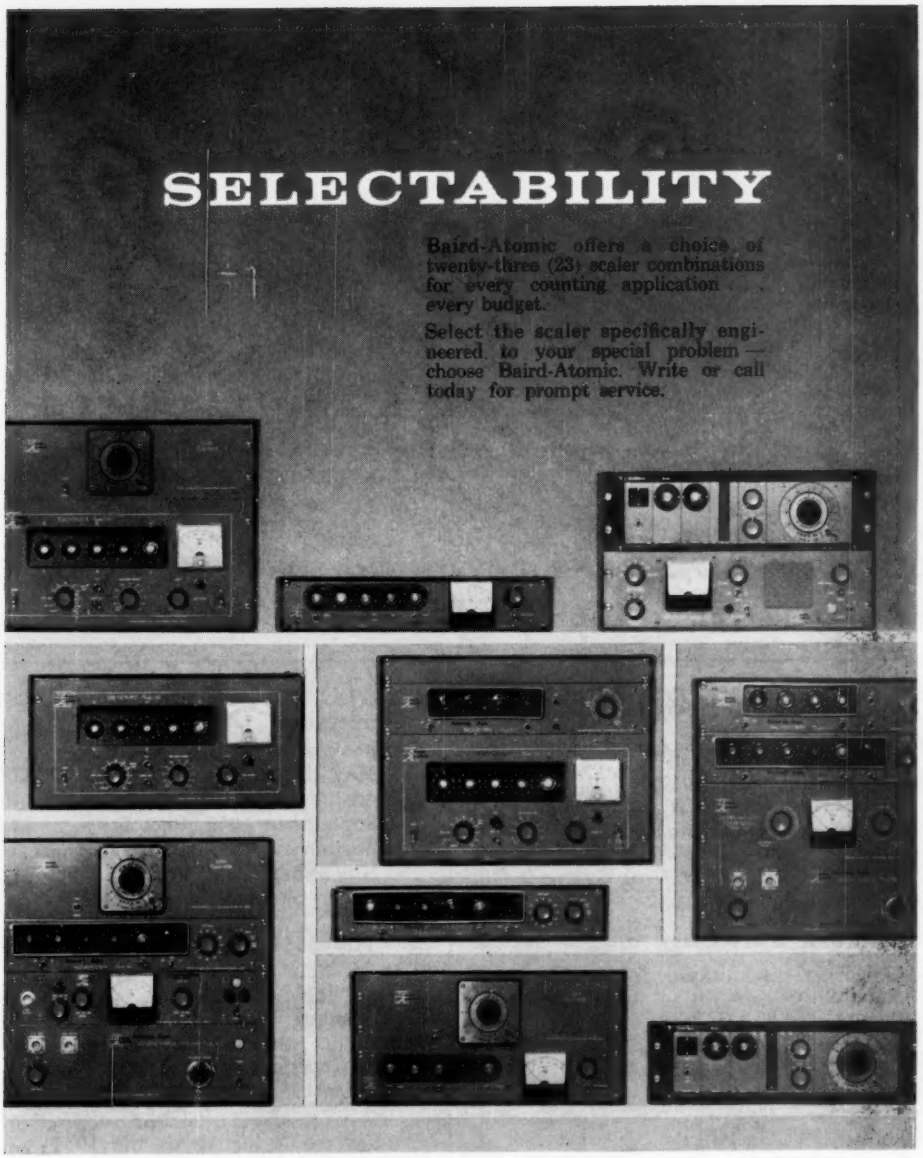
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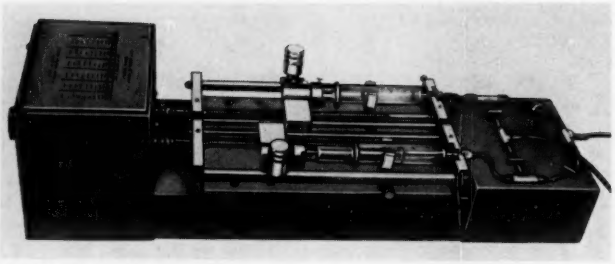

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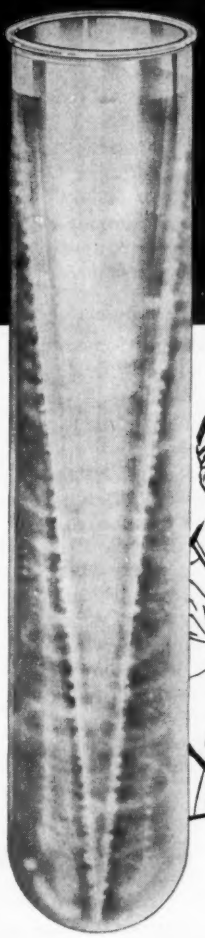

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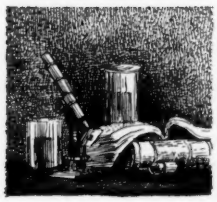

IN SEPTEMBER – (1831) – at the first annual meeting of the British Association for the Advancement of Science, John Dalton[1] reports a series of experiments concerning the relationship of his food consumption to his body secretions. He shows that the carbon content of his diet is 11½ oz. daily, of which there passes off sensibly 1 oz. The carbonic acid gas expelled from his lungs contains 10¼ oz. of carbon leaving ¼ oz. lost as insensible skin perspiration. Mr. Dalton's "regular habits of life and uniform good health" added validity to this self-experimentation.

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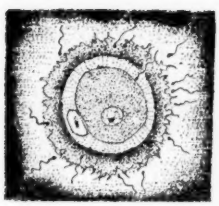

IN SEPTEMBER – (1910) – speculation concerning the chemical basis of heredity continues. In an address to the British Association Section on Zoology, G. C. Bourne states, "All the evidence at our disposal goes to show that the potentialities of germ-cells are determined at the close of the maturation divisions. Following the physiological line of argument, it must be allowed that in this connection 'potentiality' can mean nothing else than chemical constitution. If we admit this, we admit the validity of the theory, advanced by more than one physiologist, that heritable 'characters' or 'tendencies' must be identified with the enzymes carried in the germ cells."[3]

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1. Dalton, J.: On the proportion between the quantity of food and the evacuations. Rep. Brit. A. Adv. Sc., 1831, p. 74. 2. Prentiss, D. W.: Pilocarpin – its action in changing the color of the human hair. Science *2*:442 (Sept. 17) 1881. 3. Bourne, G. C.: Address to The British Association at Sheffield. Nature *84*:378 (Sept. 22) 1910.

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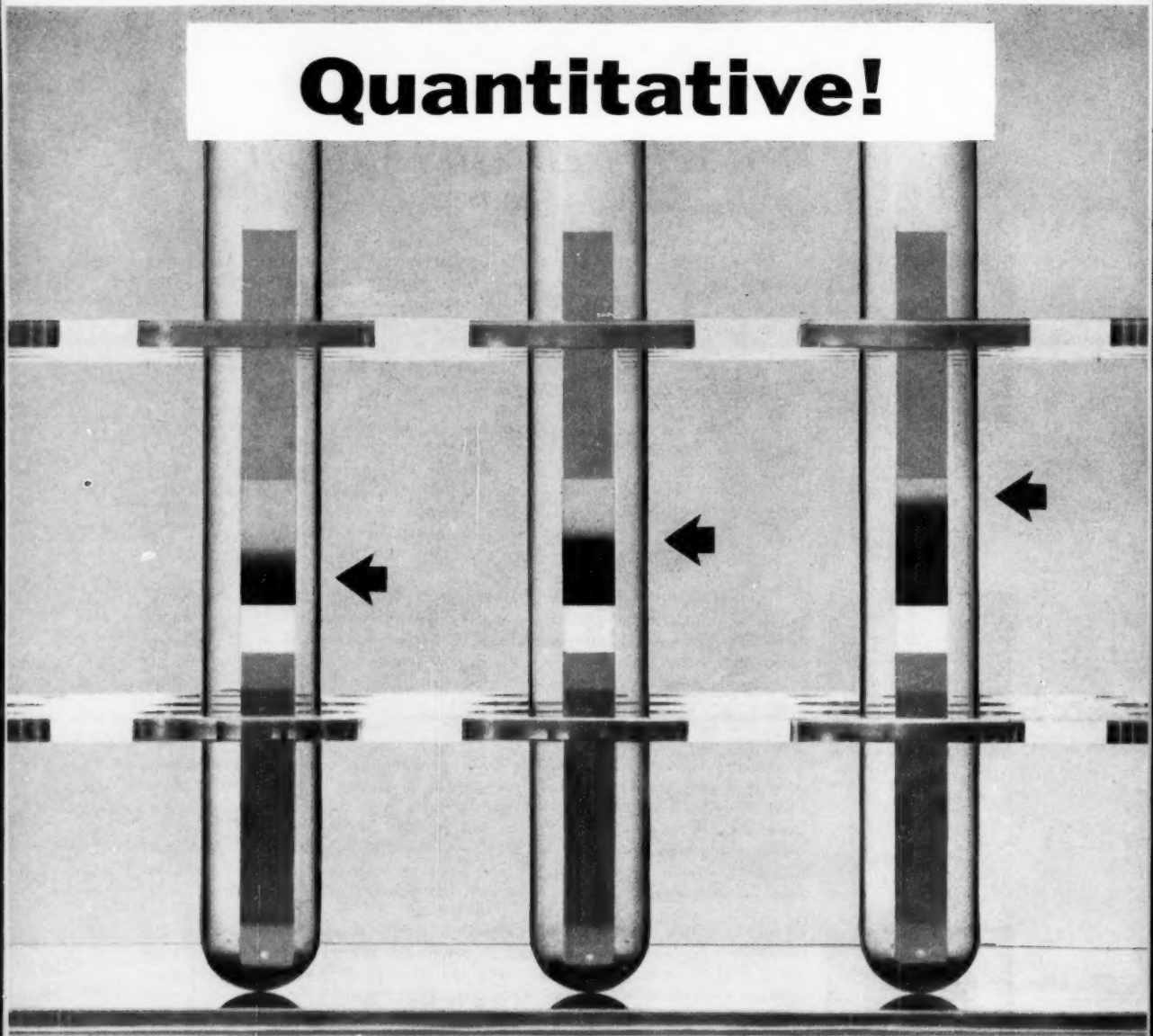

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THE EFFECTS OF BODY POSITION, GANGLIONIC BLOCKADE AND NOREPINEPHRINE ON THE PULMONARY CAPILLARY BED *†

By BENJAMIN M. LEWIS, WILLIAM T. McELROY,‡ ERNEST J. HAYFORD-WELSING§ AND L. CARL SAMBERG

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(Submitted for publication February 17, 1960; accepted April 7, 1960)

Roughton and Forster (1) have demonstrated that the volume of blood in the pulmonary capillaries (V_c) and the true diffusing capacity of the pulmonary membrane (D_M), which, presumably, is related to the thickness and area of the pulmonary capillary wall, may be calculated from measurements of the pulmonary diffusing capacity for carbon monoxide ($D_{L_{CO}}$) at several different capillary oxygen tensions. Their method makes possible the study in the intact human of a capillary bed which is of great physiological and clinical importance. We have previously studied (2) the behavior of this bed in mild exercise and during change in position. In the present study, we have infused a ganglionic blocking agent and norepinephrine during change of body position and have correlated the changes which occurred in V_c and D_M with available information on the cardiovascular effects of these procedures to explore the manner in which the pulmonary capillary bed is regulated.

MATERIALS AND METHODS

Subjects for these studies were young adult male volunteers in good health. Physical characteristics of these subjects are given in Table I. In each subject, the tension of carbon monoxide in equilibrium with capillary blood was determined by a rebreathing method (3). The subject then lay on a comfortably padded tilt table and an intravenous infusion of 5 per cent dextrose in water was begun and allowed to run slowly. The experimental procedure shown in Table II was then carried out.

The infusion solution was then changed to either trimethapan camphor-sulfonate (Arfonad), a ganglionic blocking agent (5), 1 mg per ml, or norepinephrine (Levophed), 4 μg per ml. The rate of infusion of trimethapan was regulated so that the systolic blood pressure after one minute in the 45° head-up position was approximately 90 mm Hg; the rate of infusion of norepinephrine was regulated to maintain a systolic blood pressure of 140 mm Hg in the recumbent position. The experimental procedure given above was then repeated. In all studies in which trimethapan was used, the infusion was discontinued and a final equilibrium P_{CO} was measured. Initial and final measurements of equilibrium P_{CO} were used in computing $D_{L_{CO}}$ (2). In four of these subjects another control determination and study during the infusion of norepinephrine were carried out. In two subjects in whom norepinephrine was infused first, the infusion fluid was changed to trimethapan and a third set of measurements made under these conditions.

The P_{O_2} of each of the rebreathing bags was measured by a paramagnetic oxygen analyzer.[1] The reciprocal of the reaction rate of CO with hemoglobin at this P_{O_2} ($1/\theta$) was taken from the published graphs of Roughton, Forster and Cander (6) assuming λ of 2.5, and the V_c and D_M in each position were obtained graphically from the plot of $1/\theta$ against $1/D_{L_{CO}}$.

TABLE I

Physical characteristics of subjects

Subject	Age	Ht	Wt	Vital capacity
	yrs	*cm*	*kg*	*L*
A. L.	24	180	73	6.1
E. H.	23	183	86	4.0
C. L.	31	180	73	2.9
D. J.	22	180	76	5.2
M. N.	23	175	77	4.6
N. Z.	35	171	80	4.4
J. L.	23	172	76	3.7
K. T.	22	178	64	4.6

* The material was presented in part at the fall meeting of the American Physiological Society, London, Ontario, Sept. 5, 1958 and at the Midwestern Section of the American Federation for Clinical Research, Chicago, Ill., Oct. 30, 1958.

† This work was supported by grants from the National Heart Institute (H2379), the Receiving Hospital Research Corp., and the Michigan Heart Association.

‡ This work was performed during an American Physiological Society Summer Research Traineeship.

§ This work was performed during tenure of a Fellowship with the Michigan Heart Association.

[1] Model E2, Beckman Instruments, Inc., Fullerton, Calif.

TABLE II

Plan of experiment

Time		Position	Gas breathed	Measurements*
min	*sec*			
0	0	Recumbent	Oxygen	
2	0	45° Head-up	Oxygen	
3	0	Recumbent	Oxygen	
4	0	Recumbent	0.3% CO, 10% He, 89.7% O_2†	D_{LCO}, blood pressure, pulse
4	30	Recumbent	Oxygen	
6	30	45° Head-up	Oxygen	
7	30	45° Head-up	0.3% CO, 10% He, 89.7% O_2†	D_{LCO}, blood pressure, pulse
8	0	Recumbent	Room air	
11	0	45° Head-up	Room air	
12	0	Recumbent	Room air	
13	0	Recumbent	0.3% CO, 10% He, 21% O_2, 68.7% N_2†	D_{LCO}, blood pressure, pulse
13	30	Recumbent	Room air	
15	30	45° Head-up	Room air	
16	30	45° Head-up	0.3% CO, 10% He, 21% O_2, 68.7% N_2†	D_{LCO}, blood pressure, pulse
17	0	Recumbent	Room air	

* D_{LCO} was measured by a rebreathing method (4).
† Obtained from The Matheson Company, East Rutherford, N. J.

RESULTS

The results obtained are shown in Table III. These data are summarized in Table IV. Arrows indicate significant differences when the data are subjected to variance analysis (7). In the trimethapan group, studies on D. J. and J. L. are included although norepinephrine was infused before trimethapan was given. The changes following trimethapan in these subjects are of approximately the same magnitude and direction as in those in whom trimethapan infusion immediately followed the control. The direction of changes from the recumbent position during the various procedures employed is indicated in Table V.

DISCUSSION

Limitations of the methods. We have discussed elsewhere the accuracy of the rebreathing method of measuring D_{LCO} used in these studies (4) and of the computation of V_c and D_M (2).

With regard to the rebreathing method, we concluded that the values obtained by its use are less subject to errors due to uneven ratios of diffusing capacity to alveolar volume or to alveolar ventilation than other methods using carbon monoxide. Further, any changes in the volume of the lung during the procedures used in these studies are taken account of, since a measurement of the combined volume of lung and rebreathing bag is obtained simultaneously with a measurement of D_{LCO}.

With regard to the computation of V_c and D_M, we felt that the values obtained were approximate rather than exact and that the chief usefulness of such computations lay in detecting changes in these parameters. The fact that the values of V_c and D_M that satisfy a given value of D_{LCO} lie on a rectangular hyperbola must be borne in mind in assessing the significance of such changes. Because of this relation, it is possible that a change in D_{LCO} within the limits of experimental error might cause a striking change in the computed values of V_c or D_M. Standard statistical methods, such as those used in this study, should distinguish such random changes from significant alterations. In addition, we may compare data on the effects of tilting in the present study with data we have previously reported (2) on changes in V_c and D_M in the sitting and recumbent positions using a different method of measuring D_{LCO} (Table VI). The absolute values for D_{LCO} and V_c differ, especially the latter, but the direction of change is the same. D_M increased in the sitting position in the single breath studies, but did not change in the rebreathing data. However, the

TABLE III

*Measurements of diffusing capacity, capillary volume and membrane diffusing capacity**

Subject	Position	Drug	D_{LCO} Po_2 100	D_{LCO} Po_2 600	V_c	D_M	Blood pressure	Pulse
			ml/mm Hg × min		*ml*	*ml/mm Hg × min*	*mm Hg*	
A. L.	R	N	39.6	26.5	211	52	113/67	57
	T	N	36.5	20.4	125	60	100/73	71
	R	A	38.8	23.2	153	60	101/64	67
	T	A	38.6	19.8	107	84	102/74	79
	R	N	35.8	22.6	175	50	105/61	66
	T	N	37.5	19.1	107	83	103/74	85
	R	L	44.5	23.1	137	99	140/81	56
	T	L	43.8	23.5	151	72	141/82	66
E. H.	R	N	37.4	24.6	176	52	120/80	58
	T	N	34.7	21.2	145	54	118/92	59
	R	L	42.4	25.4	182	64	143/86	59
	T	L	38.2	23.4	167	55	141/87	53
C. L.	R	N	32.0	19.9	143	47	130/100	
	T	N	24.0	15.0	120	33		
	R	A	30.3	15.3	83	62	137/92	
	T	A	22.0	12.0	69	41	80/57	
D. J.	R	N	39.1	21.7	143	64	120/170	70
	T	N	37.1	19.8	119	69	112/68	76
	R	L	48.5	27.0	159	84	128/85	57
	T	L	36.4	21.7	154	55	140/91	68
	R	A	35.5	18.4	105	67	100/71	71
	T	A	30.9	15.3	87	62	86/74	102
M. N.	R	N	31.8	18.4	109	59	118/78	77
	T	N	28.1	16.8	112	45	119/88	88
	R	A	29.2	17.5	119	45	115/82	101
	T	A	26.3	15.2	95	45	101/90	122
	R	N	29.0	17.8	125	44	124/78	81
	T	N	28.7	16.4	107	47	124/88	98
	R	L	32.8	18.9	135	48	151/99	76
	T	L	29.5	17.5	132	42	142/91	79
N. Z.	R	N	24.1	15.2	114	35	106/80	60
	T	N	22.6	12.6	79	35	106/82	62
	R	A	21.4	14.1	114	29	100/80	62
	T	A	18.7	11.1	77	28	90/80	82
	R	N	21.2	14.7	139	27	111/74	66
	T	N	19.8	12.2	85	30	107/75	64
	R	L	24.0	15.9	128	32	144/93	49
	T	L	26.0	16.2	119	38	136/85	54
J. L.	R	N	23.6	14.5	95	37	110/65	75
	T	N	21.0	13.9	104	30	108/83	84
	R	L	23.0	14.8	108	33	140/100	48
	T	L	23.0	15.6	130	31	140/93	59
	R	A	20.3	13.2	100	28	102/72	84
	T	A	19.6	11.2	71	35	70/58	120
K. T.	R	N	34.8	20.1	130	59	108/64	65
	T	N	34.3	18.4	111	62	102/77	71
	R	A	36.0	19.6	122	57	98/68	63
	T	A	30.8	15.3	90	55	78/66	87
	R	N	34.8	21.1	144	55	107/65	63
	T	N	33.0	17.9	113	58	102/75	70
	R	L	39.2	22.8	150	65	146/89	51
	T	L	34.0	21.8	158	51	142/83	50

* The following abbreviations are used: R, recumbent; T, tilted 45°; N, glucose infusion; A, trimethapan (Arfonad) infusion; L, norepinephrine (Levophed) infusion; D_{LCO}, diffusing capacity of lungs; p_{O_2}, approximate alveolar oxygen tension; V_c, capillary blood volume; D_M, membrane diffusing capacity.

TABLE IV

Summary of results *

Condition	D_{LCO} P_{O_2} 100 R	D_{LCO} P_{O_2} 100 T	D_{LCO} P_{O_2} 600 R	D_{LCO} P_{O_2} 600 T	V_c R	V_c T	D_M R	D_M T
Control	32.1	29.1	19.5	16.7	135	110	51	48
Trimethapan	30.2	26.7	17.3	14.3	114	85	50	51
Control	31.5	30.2	19.6	17.2	143	111	47	53
Norepinephrine	36.3	33.0	21.1	19.9	143	144	61	49

* Abbreviations are the same as in Table III. Arrows indicate significant differences.

TABLE V

Relation of V_c and D_M to hemodynamic changes *

Position	Procedure	PAP	CO	PCP	PVR	TMP	V_c	D_M	Reference no.
Tilted	None	↓	↓	↓	↑	↓	↓	0	(8)
Recumbent	Trimethapan	↓	↓	[↓]	[0]	↓	↓	0	(9, 10)
Tilted	Trimethapan	[↓↓]	[↓↓]			↓↓	↓↓	0	(11)
Recumbent	Exercise	↑	↑	0	0	↑	↑	↑	(2, 12)
Recumbent	Norepinephrine	↑	0	↑	?	↑	0	↑	(13, 14)
Tilted	Norepinephrine		[0]			[↑]	0	0	Text

* Abbreviations: PAP = pulmonary artery pressure; CO = cardiac output; PCP = pulmonary "capillary" (or wedge) pressure; PVR = pulmonary vascular resistance; TMP = transmural pressure in capillary (deduced, see text); blank = no data; ? = conflicting results. Arrows indicate direction of change from recumbent position, no drug administered. For other comment see text.

conclusion drawn from the two sets of data is the same. In each case there is an increase in surface area and/or decrease in diffusion path per unit of capillary volume. This comparison strengthens our confidence in the *qualitative* validity of the changes found in these studies.

Interpretation of results. Physiological study of the capillary circulation of the lung has been made directly by biomicroscopy and indirectly by the techniques used in this study. The available data on the behavior of the pulmonary capillaries using the indirect technique are summarized in Table V.

The pulmonary capillary bed may either passively conform to events elsewhere in the cardiovascular system or possess active vasomotion. That is, the observed changes in this bed may be either secondary or primary.

The notion of a passive or secondary change in the pulmonary capillary bed needs some clarification. For a vessel which contains muscular and fibrous elements in its wall (as arterioles and venules), the criteria for such a change have been succinctly stated by Burton (15): The size of a vessel is governed by the pressure difference be-

TABLE VI

Effects of position; comparison of methods *

Method	Subjects	D_{LCO} P_{O_2} 100 R	D_{LCO} P_{O_2} 100 T	D_{LCO} P_{O_2} 600 R	D_{LCO} P_{O_2} 600 T	V_c R	V_c T	D_M R	D_M T
Single breath	4	31.6	26.9	15.0	11.5	86	59	77	99
Rebreathing	7	32.1	29.1	19.5	16.7	135	110	51	48

* Abbreviations are the same as in Table III. "Single breath" results were previously published (2) and were done in sitting rather than tilted position.

tween its lumen and the surrounding tissue (transmural pressure) and by the tension in its wall. If the size changes in the same direction as the transmural pressure, this change may be (but *not* must be) passive; if size and transmural pressure change in opposite directions, active vasomotion is involved. Capillaries, however, are simple endothelial tubes and on biophysical grounds should open fully if the transmural pressure exceeds a certain value (critical opening pressure); no gradations in patency between closed and fully open should occur (16). The remarkable constancy of pulmonary capillary diameter seen by biomicroscopy (17) confirms this prediction. To call a change in the pulmonary capillaries passive we must assume that the critical closing pressures of the pulmonary capillaries cover a range of values from low to high and that these critical closing pressures do not alter during the change in question. Then, during a rise in transmural pressure the critical opening pressure of certain capillaries previously closed should be exceeded and these capillaries will open, increasing V_c (or vice versa during a fall of transmural pressure). If these assumptions are not proved, active vasomotion cannot be excluded. On the other hand, if transmural pressure and V_c change in opposite directions, the change cannot be explained on a passive or secondary basis and active vasomotion is involved.

We are not aware of measurements of pulmonary capillary transmural pressure. However, the mean capillary transmural pressure must bear some relation to the pressure in the pulmonary artery and the pulmonary veins.

In Table V we have summarized the data available on the changes in the pulmonary circulation during the procedures we employed and our deductions from this data as to changes in the capillary transmural pressure. Transmural pressure must, of course, exceed pulmonary venous pressure and, barring venous constriction, these two pressures must change together. Pulmonary "capillary" (or wedge) pressure is an acceptable measure of pulmonary venous pressure in man (18), and in Table V we have used it as an index of changes in transmural pressure. Pulmonary artery pressure is a less reliable guide to changes in transmural pressure because, although it must always exceed transmural pressure, the two need not change together, since the resistance of the pulmonary arterioles is interposed.

In addition to mean pulmonary artery pressure and pulmonary arteriolar resistance, the transmural pressure in the capillaries may be affected by changes in the vertical distance from the capillaries to the root of the pulmonary artery during changes in position. We believe this last factor is of less importance than the other two since its effects tend to cancel out. The anterior portions of the lungs are supplied against gravity in the recumbent position while the superior portions are similarly affected in the erect position. The well known decrease of oxygen uptake in the upper lobes when the subject is upright (19) has as its corollary an increase in oxygen uptake in the lower lobes.

Certain comments on Table V should be made here. We have not found measurements of pulmonary "capillary" pressure during trimethapan administration and have used the data of Fowler and associates (10) for a related drug, tetra-ethyl ammonium. Fowler (11) does not specify the ganglionic blocking agent used in his studies. The data on pulmonary vascular resistance during norepinephrine administration are conflicting (13, 14), but the conclusion that capillary transmural pressure rises seems warranted, regardless of change in pulmonary vascular resistance. The data on the effects of norepinephrine infusion during head-up tilt are scanty. DeFazio, Regan and Binak [2] have found that cardiac output did not change when a patient was tilted during norepinephrine infusion. The secretion of norepinephrine is a normal response to assuming the upright position (20) and deficiency of such secretion has been reported in orthostatic hypotension (21). Presumably, norepinephrine infusion prevents venous pooling and thus maintains cardiac output, pulmonary artery pressure and transmural pressure at or above control values. The data which have been specificially mentioned above are indicated by brackets in Table V.

It will be seen that during tilting, trimethapan infusion and exercise, transmural pressure and V_c change in the same direction. These changes

[2] Personal communication.

might be passive or secondary to the variation in transmural pressure brought about by events elsewhere in the cardiovascular system. However, during norepinephrine infusion in the recumbent position in which hemodynamic data suggest that capillary transmural pressure increases, V_c does not change. This is evidence in favor of active vasomotion in the pulmonary capillary bed.

The possible site of such vasomotion is of interest. In general, arterioles are joined to venules by thoroughfare or preferential channels which have thin muscular walls and are direct continuations of the feeding arteriole. True capillaries arise as lateral branches of these thoroughfare channels (*22*). Garcia-Ramos (*17*) has concluded that this arrangement of thoroughfare channels with lateral branches exists in the lungs. A ring of smooth muscle (precapillary sphincter) is known to exist at the junction of true capillaries with their thoroughfare channels in other organs and such sphincters are exquisitely sensitive to circulating vasoconstrictors like norepinephrine (*22*). If such sphincters existed in the lung, their constriction might prevent the increased transmural pressure from opening previously closed capillaries.

It should be reiterated at this point that because a change in capillary volume *could* be explained by a passive conformity of the capillary bed to a change in transmural pressure does not mean that this is the mechanism involved. The occurrence of capillary vasomotion is not excluded in certain of these situations. Thus, if the norepinephrine secreted during tilting (*20*) were to constrict the precapillary sphincters, capillary volume might decrease independently of changes in transmural pressure. However, capillary volume goes up on exercise when norepinephrine release also occurs (*23*) and trimethapan, which should block norepinephrine release, causes a more profound drop of capillary volume on tilting, not a less profound change as one might predict if one views capillary vasomotion as the mechanism governing the capillary bed. This, of course, assumes that trimethapan has no action of its own on the precapillary sphincters as shown in the rat mesentery (*24*).

Thus, from the limited evidence available, the changes in capillary volume which occur on tilting, during exercise and during trimethapan infusion can be more readily explained as passive responses of the capillary bed to events elsewhere in the cardiovascular system, which vary transmural pressure, than as active vasomotion of this bed. However, the fact that capillary volume does not increase during norepinephrine infusion suggests that under certain circumstances active vasomotion does occur in the pulmonary capillary bed. It is possible that both secondary and primary effects on the capillary bed occur in most situations, but more subtle experiments involving perfusion and quantitative biomicroscopy would be necessary to detect this interaction.

Interpretation of the changes in D_M in these studies is more complex than that of the changes in V_c. D_M presumably is a measure of the surface area of the capillaries in contact with the alveoli and of the distance that gas must travel from alveolus to capillary. Either or both of these parameters may change.

According to the Starling hypothesis, in a "solid" organ capillary transmural pressure, the osmotic pressure of the blood, and tissue pressure are in equilibrium. If transmural pressure rises, fluid passes from the capillary until tissue pressure increases to restore equilibrium. The process is reversed if transmural pressure falls. It is not known if significant tissue pressure can develop in this way between the alveolar and capillary walls in a "spongy" organ like the lung or if fluid passing from the capillary remains within this wall, thickening it. If this latter process does occur, a fall in transmural pressure would cause fluid to enter the capillary and decrease the distance for diffusion. This is a possible explanation of the finding that D_M did not change during tilting or trimethapan infusion, which decrease transmural pressure. During exercise both D_M and transmural pressure increase. V_c also increases, however, indicating that more capillaries are open with greater surface area. The increase in D_M during norepinephrine infusion remains unexplained.

Measurement of changes in lung tissue volume (*25*) concurrently with D_M might place interpretations of D_M on a sounder footing. Thus, if lung tissue volume were to fall on tilting, although D_M did not change, there might be some support for the speculation advanced above that this is due to

passage of fluid from the alveolar capillary space into the capillary.

SUMMARY

1. The pulmonary capillary blood volume (V_c) and the diffusing capacity of the pulmonary membrane (D_M) were calculated from measurements of diffusing capacity of the lungs for carbon monoxide ($D_{L_{CO}}$) in normal subjects in the recumbent and 45° head-up tilted positions, during a control period and during the infusion of trimethapan (Arfonad) and norepinephrine (Levophed).

2. V_c fell during head-up tilting. The infusion of trimethapan in the recumbent position also decreased V_c and accentuated its decrease during the head-up tilt. D_M did not change significantly during these procedures.

3. Norepinephrine did not change V_c in the recumbent position, but led to an increase in D_M. The decrease in V_c on tilting was abolished by norepinephrine.

4. V_c changed in the same direction as did the capillary transmural pressure deduced from available hemodynamic data during tilting, trimethapan infusion, and exercise. This could be due to passive conformity of the capillary bed to changes in transmural pressure. Active vasomotion, however, cannot be excluded. V_c did not change, although transmural pressure probably was increased during norepinephrine infusion in the recumbent position. This is best explained by active vasomotion in the pulmonary capillaries.

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THE LOCAL EXUDATIVE CELLULAR RESPONSE IN LEUKEMIA *

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(Submitted for publication March 22, 1960; accepted April 22, 1960)

The present study was undertaken to examine the local exudative cellular response in patients with leukemia and to determine whether alterations in the pattern of response might further explain their increased susceptibility to infection. Portions of the complex process of host resistance have been shown to be abnormal in leukemia (1–4). Some studies have demonstrated reduced phagocytic activity of the neutrophilic granulocytes in chronic granulocytic leukemia (3, 4), but this opinion is not universally shared (5). Although natural antibody levels appear to be maintained in leukemia (6), impaired *de novo* antibody synthesis has been demonstrated in chronic lymphocytic leukemia (1, 2). Despite the fundamental role of the inflammatory reaction in determining the body's resistance to bacterial invasion, the characteristics of this important defense mechanism have received little attention in leukemia.

MATERIALS AND METHODS

A group of 26 patients with various types of acute and chronic leukemia and 10 hematologically normal subjects were included in this study. The normal subjects were adults admitted to the hospital with a variety of disorders, none of which would be expected to interfere with the local inflammatory response.

Of the 26 patients with leukemia, 20 were male and 6 were female. Their ages ranged from 18 to 74. Most of the chronic leukemia patients were in adequate hematological remission and were receiving maintenance chemotherapy, while most of the acute leukemia patients were in an active phase of their disease (Table I).

The method employed was a modification of the glass coverslip technique developed by Rebuck and Crowley and described in detail elsewhere (7). The skin over a small area of the volar surface of the forearm was scraped with a sterile scalpel until the papillary layer of the corium was exposed. The lesion then was covered with a sterile glass coverslip which in turn was surmounted by a sterile cardboard square and 2 inch surgical tape. The cells responding to the combined stimulation of the surgical abrasion and glass surface adhered to the undersurface of the coverslip. The coverslips were removed at timed intervals, quickly air-dried, stained with Wright-Giemsa and mounted on glass slides.

The slides were then examined microscopically. Each preparation was carefully examined with a low power scanning lens and its relative cellularity determined. At each stage of the local exudative cellular reaction the normal responses were graded as showing maximum cellularity (4+). Each leukemic preparation was then graded 0 to 4+ in comparison to the normal response at a comparable stage of the inflammatory cycle. The morphology and maturity of the local cellular exudates were determined under an oil immersion lens. Serial coverslip preparations were obtained at hourly intervals from each lesion for the first 12 to 14 hours and at 22 to 24 hours after the initiation of the inflammatory

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TABLE I

Clinical description of patients studied

Type of leukemia	WBC at time of study $\times 10^3$/mm^3			Therapy at time of study					Total patients studied
	<5	5 to 20	>20	None	Busulphan	Chlorambucil	6-Mercaptopurine	Steroid	
Chronic granulocytic		5	1	1	5				6
Chronic lymphocytic		8	2	3		7		2	10*
Acute granulocytic		2	1	2			1	1	3*
Acute lymphocytic	2	1						3	3
Acute monocytic		2	2				2	4	4*
								Total	26

* Some patients were receiving combined therapy.

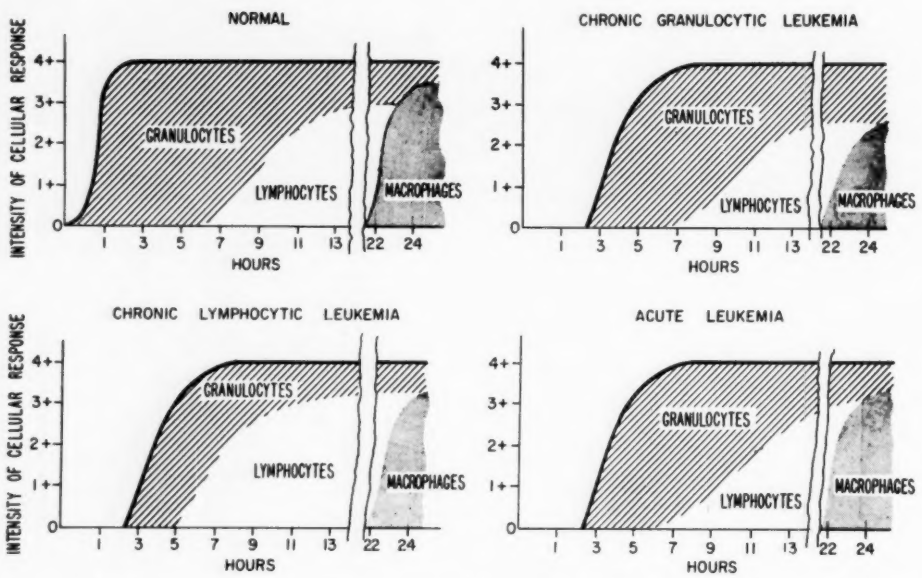


FIG. 1. GRAPHIC REPRESENTATION OF THE LOCAL CELLULAR RESPONSE IN NORMAL SUBJECTS AND IN PATIENTS WITH LEUKEMIA. The heavy line represents the intensity of the local cellular responses graded 0 to 4+ (see text). The area below the heavy line is partitioned to show the time of initial appearance and relative participation of the three main types of responding cells during the 24 hour period studied.

response. Two lesions were studied on the forearm of each patient and control subject.

RESULTS

Local cellular response in normal subjects (Figure 1). In general our findings in the normal subjects agreed with the earlier reports of Rebuck and Crowley (*7*). At about 1 to 1.5 hours following injury a heavy concentration of mature neutrophilic granulocytes was noted on the coverslip. This phase persisted for the first 6 to 8 hours of the inflammatory cycle. During this period there was minor participation by tissue histiocytes, eosinophils and lymphocytes. At the eighth to tenth hour of the cycle large, mature lymphocytes began appearing in increasing numbers and by the fourteenth hour they made up about 50 per cent of the local cellular exudate. From this point in the inflammatory cycle an increasing number of large, sessile macrophages was noted so that by the twenty-second to twenty-fourth hour they comprised over 80 per cent of the cells present. Rebuck and Crowley have shown these cells to be derived from the lymphocytes present earlier and from tissue histiocytes (*7*).

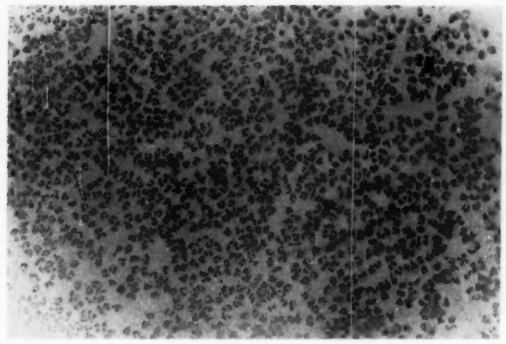
Local cellular response in leukemic patients (Figure 1). The local cellular response in patients with leukemia differed in several ways from the normal. In all patients studied the initial neutrophilic granulocyte response was delayed in its onset, beginning usually 3 to 3.5 hours following injury. In comparison with the normal this initial response was sparse, and maximum cellularity developed more slowly (Figure 2).

In most of the patients with chronic granulocytic leukemia, granulocytes persisted in significant numbers throughout the 24 hour period. Of additional interest was the participation of significant numbers of basophils in the local response of these patients (Figure 3).

In chronic lymphocytic leukemia increasing numbers of mature lymphocytes began appearing at the fifth to seventh hour, whereas in the normal subjects this phenomenon did not begin until the eighth to tenth hour of the local cellular response (Figure 4).

Except for the altered initial granulocyte response the patients with acute leukemia demonstrated no characteristic cellular patterns.

In general the local exudative responses were



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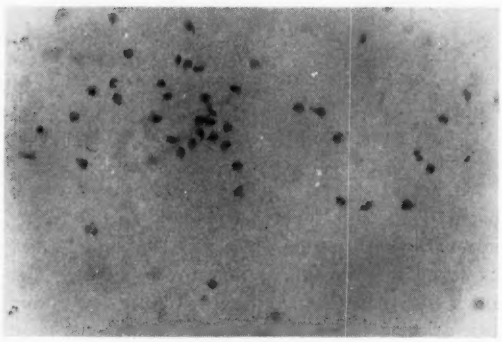

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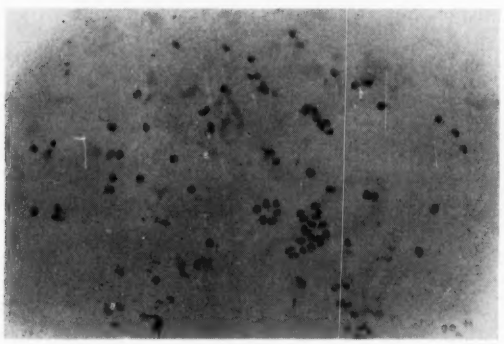

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FIG. 2. FOREARM COVERSLIP PREPARATIONS AT THE THIRD HOUR OF THE LOCAL INFLAMMATORY CYCLE SHOWING THE MORPHOLOGY AND INTENSITY OF THE INFLAMMATORY RESPONSE. A) Normal (4+); B) chronic granulocytic leukemia (1+); C) acute lymphoblastic leukemia (1+) (×1,200).

made up primarily of morphologically mature cells regardless of the morphology and cellularity of the peripheral blood or bone marrow. This was most strikingly demonstrated in the patients with acute leukemia (Figure 5). In only one patient in this latter group were a few nucleolated cells of histiocytic type seen in the late stages of the local exudative response. Two patients with acute subleukemic leukemia showed no cellular response in the 24 hour period studied. The characteristics of the inflammatory responses observed in these patients had no relationship to either the type of therapy or degree of hematological remission. Even those patients with chronic leukemia whose total leukocyte counts were within the normal range showed the delayed granulocyte response in the local inflammatory reaction. Neither age nor sex appeared to have a significant effect on the responses described.

DISCUSSION

The morphologic characteristics of the inflammatory exudate were easily reproducible. In both groups the time sequence for the appearance of particular cell types showed little variation from patient to patient. The injury produced by the abrasion and the continued application of the coverslips was sufficient to reproduce the complete inflammatory cycle described by Rebuck and Crowley using specific nonpyrogenic bacterial substances (7). The leukocyte preparations obtained were well preserved permitting accurate identification of cellular morphology and maturity. Despite limitations in quantitation, the decreased intensity of the early granulocytic response in the leukemic patient was so obvious that it is believed to be significant.

The results of these experiments demonstrate that the early exudative cellular response of inflammation in various types of leukemia is both delayed and diminished in comparison to the normal. Earlier studies of the local inflammatory reaction have shown the first few hours of this response to be the crucial period which determines the success or failure of microbial invasion (8). The neutrophilic granulocyte is considered the cell of major importance during this phase of the body's defense and its prompt arrival in adequate numbers at the site of injury constitutes the first line of defense against such invasion. The delayed and diminished granulocyte response demonstrated in our patients may provide a more adequate explanation for the susceptibility of leukemic patients to infection. It is interesting to note that similar

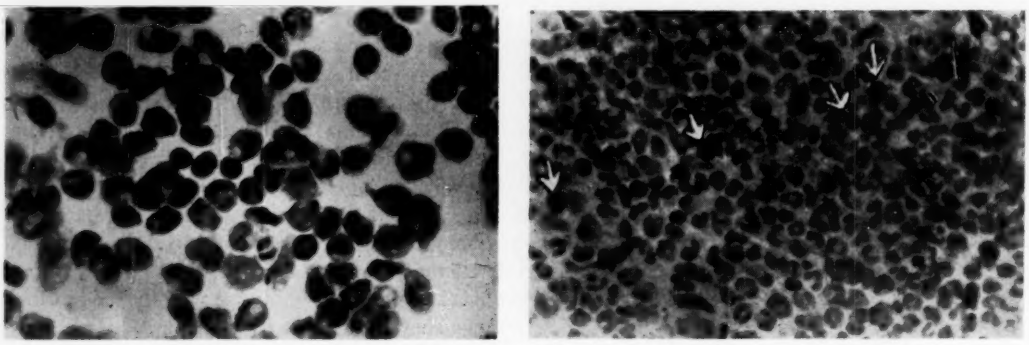

FIG. 3. LOCAL INFLAMMATORY REACTION IN CHRONIC GRANULOCYTIC LEUKEMIA (RIGHT) (× 1,500) AND IN NORMAL SUBJECTS (LEFT) (× 1,600) AT THE TWENTY-SECOND HOUR OF INFLAMMATION. Several of the numerous basophils present are marked (arrows).

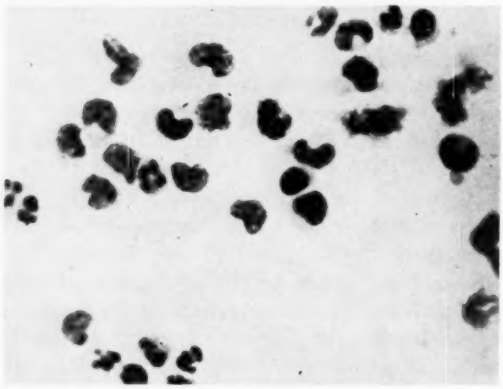

FIG. 4. A 5.5 HOUR PREPARATION IN CHRONIC LYMPHOCYTIC LEUKEMIA SHOWING THE PRESENCE OF SIGNIFICANT NUMBERS OF LYMPHOCYTES. These cells are frequently fixed in ameboid motion accounting for their irregular cellular outlines and the exaggerated indentations of the nuclei (× 1,600).

defects in the local defense reaction have been demonstrated in diabetic rabbits (9). These results also were interpreted as indicating increased susceptibility to infection.

Dionisi (10) and Bickhardt (11) have stated that patients with leukemia respond to inflammation with immature leukemic cells. Except for the atypical and somewhat immature appearing cells which were noted late in the response of one of our patients with acute leukemia, we found, as Jaffe did, that the patients with leukemia responded to inflammation with morphologically mature cells (12).

The appearance of heavy concentrations of mature polymorphonuclear leukocytes on the coverslip preparations of the patients with chronic lymphocytic leukemia was in striking contrast to the differential leukocyte counts of the peripheral blood. In spite of the preponderance of circulating

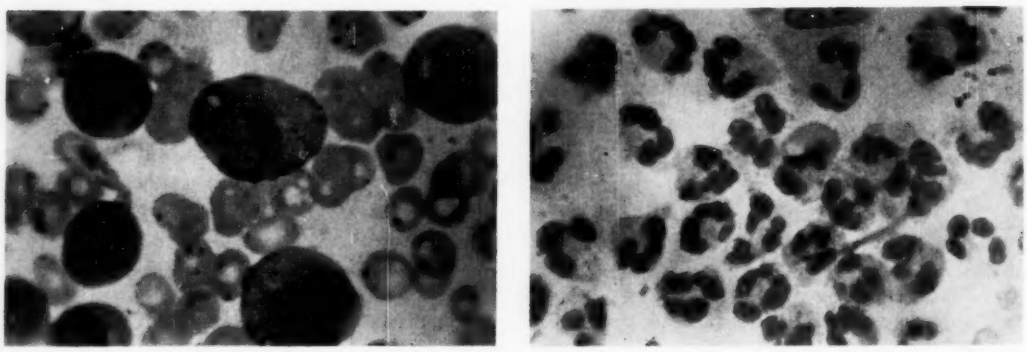

FIG. 5. FIVE HOUR COVERSLIP PREPARATION (RIGHT) AND PERIPHERAL BLOOD SMEAR (LEFT) IN A PATIENT WITH ACUTE MONOCYTIC LEUKEMIA CONTRASTING THE MATURITY OF THE LOCAL CELLULAR RESPONSE TO THE IMMATURITY OF THE PERIPHERAL BLOOD (× 2,000).

lymphocytes in these patients, the number of circulating mature granulocytes usually was within normal limits. It is well known that purulent exudates in patients with chronic lymphocytic leukemia consist primarily of polymorphonuclear leukocytes.

Others have shown that the degree of participation of specific mature cells in local inflammatory sites is in general related to their numbers in the circulating blood (13). This relationship was demonstrated by the preponderance of neutrophilic and basophilic granulocytes throughout the 24 hour period in the patients with chronic granulocytic leukemia and the early appearance of lymphocytes in most of the patients with chronic lymphocytic leukemia.

Jaffe has stated that some myeloid tissue capable of maturation must be present in leukemic patients if the cellular response to inflammation is to occur (12). The failure of two of our patients with acute subleukemic leukemia to show any local exudative cellular response to injury during the period studied could be explained in this manner. Both had peripheral white cell counts below 1,000 per cu ml consisting almost entirely of blast cells. In addition, repeated attempts at marrow aspirations were unsuccessful and trephine specimens demonstrated hypocellular, blastic marrows.

We have found the method used in this study a reliable, safe and simple one which lends itself to clinical applications. It may be useful in evaluating the exudative cellular aspects of the local inflammatory response in other disease states.

SUMMARY AND CONCLUSIONS

In leukemia, the initial granulocyte response to inflammation is both delayed and diminished. This feature of the exudative cellular response probably plays a major role in the susceptibility of these patients to infection. In general, the local exudative cellular response in leukemia is comprised of morphologically mature cells. The technique employed should prove valuable for evaluating the local inflammatory response in other disease states.

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COMPOSITION AND PROPERTIES OF HUMAN CERVICAL MUCUS. I. ELECTROPHORETIC SEPARATION AND IDENTIFICATION OF PROTEINS *

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The importance of cervical mucus in female infertility is generally recognized (1). Clinical characteristics of human cervical mucus have been the subject of many studies in recent years. However, very little is known about the chemical composition or the biological significance of this epithelial secretion.

Cervical mucus normally contains from 92 to 94 per cent water in the pre- and post-ovulatory phases, while in midcycle the water content rises to 98 per cent (2). The primary constituent is believed to be a mucopolysaccharide composed of galactose and hexosamine as well as some fucose and sialic acid (3, 4). Recent experiments with S^{35}-labeled sulfate have shown the presence of sulfate in the cervical mucus of rabbit and guinea pigs (5, 6). Both human and rabbit cervical gland and secretions react metachromatically with toluidine blue (7–9), indicating the presence of an acid mucopolysaccharide. The absence of uronic acid and the lack of effect by testicular hyaluronidase on cervical mucus eliminates the probability that this mucopolysaccharide is either hyaluronic acid or one of the chondroitin sulfates. If the sulfate groups are bound to mucopolysaccharides, which has not been proven, it seems likely that they are from keratosulfate, a sulfated polysaccharide composed of galactose and glucosamine (9, 10).

The presence of proteins in the cervical mucus is generally conceded (3, 4, 10). Pommerenke found that cervical mucus gave a positive biuret test and positive reactions for tryptophan, tyrosine and cysteine (2). Werner determined the total nitrogen content of the pooled cervical mucus to be 12.1 per cent (4). This, in part, represented protein(s) which could be digested by trypsin, and which yielded 15 amino acids upon acid hydrolysis. Shettles, Dische and Osnos have suggested the total protein content to be about 1 per cent (3), while Werner reported between 0.5 and 3 per cent (10).

Spencer, Sunseri and Sunseri (11), using paper electrophoresis, found two main bands in cervical mucus of normal and pregnant women, a nonmigratory band and a diffuse one. The nonmigratory band stained strongly with periodic acid-Schiff reagent (PAS), was strongly metachromatic, and positive to the Feulgen reaction. The diffuse band was PAS-positive, faintly metachromatic, and negative to the Feulgen reaction. No areas identifiable with the usual serum peaks were recognized as normal components of the cervical mucus. As a matter of fact, it was suggested that the appearance of albumin in cervical mucus would indicate the existence of some pathological state in the genital tract. Electrophoretic studies using a paper medium are open to serious question because of complications arising from the highly viscous character of the sample.

The purpose of the present study is to show the presence of albumin and other electrophoretically identifiable protein areas in the normal cervical mucus using the technique of agar gel electrophoresis.

METHODS

Collection of specimens. Samples of cervical mucus were obtained from selected patients attending the gynecological outpatient clinic at Detroit Receiving Hospital. Great care was taken to exclude all cases of menstrual disorders, vaginitis, gross cervicitis, and other pelvic abnormalities. Table I shows the clinical data for 21 cases used in this study ranging in age from 16 to 36. Eighteen women had had between 1 and 10 pregnancies, 2 women were unmarried, and 1 was newly married. The day of the cycle on which the specimen was taken was determined by questioning the patient and by correlating this date with the physical appearance of the cervical secretion, its pH, spinnbarkeit (12), and extent of arbor-

* This study was supported by Grants A-3430 and RG-7031(a) from the National Institutes of Health, Bethesda, Md.

ization (13). Cervical mucus was aspirated from the cervical canal, after gently cleaning the portio and external os with a cotton ball. All samples were refrigerated and examined within 24 hours. Specimens tinged with blood were discarded.

Electrophoretic separation. An attempt was made to separate the proteins of cervical mucus by electrophoresis on paper. In agreement with Spencer and co-workers (11), only a nonmigrating and a diffuse band were obtained, as shown in Figure 1. These authors considered such patterns typical for normal cervical mucus. Previous experience in our laboratory with synovial fluid often showed poor separations of proteins unless the samples were first treated with hyaluronidase to depolymerize the hyaluronic acid (14). Therefore, it was considered possible that the mucopolysaccharide of cervical mucus in some way might have hindered the electrophoretic separation of the proteins when a paper supporting medium was used. In a preliminary test the gelatinous character of a sample of cervical mucus was markedly reduced prior to paper electrophoresis by treating the sample with a small amount of 0.1 N NaOH. However, this did not change the electrophoretic pattern.

Electrophoresis in an agar medium was considered to be the most likely technique for separating proteins of the highly viscous samples of cervical mucus. The method of Zak, Volini, Briski and Williams was used in which only enough agar was added to the buffer medium to act as a thickening agent (15). Preliminary experiments showed that good separations of the proteins of cervical mucus could be obtained by this technique.

TABLE I

Clinical data

Case	Hospital serial no.	Age	Cycle	Gravida	Para	Remarks
1	301529	26	3/28	I	I	
2	204180	16	4/28	0	0	Unmarried
3	37890	24	5/28	II	II	
4	245688	35	3/27	III	II	
5	77701	22	4/28	III	III	
6	280845	27	5/28	II	I	Salpingectomy for ectopic pregnancy
7	18743	36	4/26	VIII	VI	Salpingectomy for ectopic pregnancy
8	67438	31	3/28	X	VI	
9	316767	23	5/28	II	II	
10	71233	35	3/28	VII	VI	
11	2283	31	7/28	V	III	
12	314959	17	4/30	I	I	
13	136310	17	4/28	0	0	Unmarried
14	682998	19	5/28	II	II	
15	310619	21	5/26	I	I	
16	304614	21	5/30	III	III	
17	315191	24	3/28	II	II	
18	118825	27	3/28	0	0	
19	101264	28	3/35	V	IV	
20	317666	19	4/30	I	I	
21	145628	21	1/35	II	II	Mucus very poor in protein*

* Protein content was too small to allow electrophoretic study.

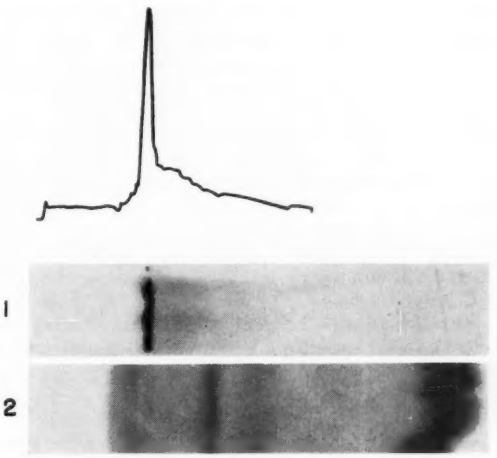


FIG. 1. AN ATTEMPTED ELECTROPHORETIC SEPARATION OF CERVICAL MUCUS PROTEINS ON WHATMAN NO. 3 MM PAPER (SPINCO APPARATUS). Strip 1, a sample of normal cervical mucus showing no migration of proteins; Strip 2, concurrently run sample of human serum showing usual electrophoretic migration of proteins.

The apparatus used for this study was identical with that described by Zak and associates (15). A watertight compartment for holding the agar-buffer medium was made by cementing [1] glass rods (2 mm in diameter) to the four edges of a 3.5 × 4 inch lantern slide coverglass which had previously been covered by a sheet of vegetable parchment paper.[2] About 15 ml of a hot agar-buffer solution, prepared by dissolving 250 mg of Bacto-Difco (certified pure) agar in 100 ml of boiling 0.05 μ veronal buffer, pH 8.6, was transferred to the glass basin. The plate was raised to the same level as the buffer in the electrode boxes by means of a micro lab-jack. Paper wicks [3] were used to connect the agar medium with the buffer boxes. The agar-buffer solution was then allowed to gel. The samples were applied by immersing small strips (0.5 × 8 mm) of Whatman no. 3 MM filter paper in the cervical mucus and by placing these on the agar gel about 1.25 inches from the cathodic end. A sample of human serum, prestained with bromphenol blue, was similarly placed on the agar as a guide for estimating the length of migration and also for comparing the position of the various fractions in the mucus specimen. After electrophoresis for 45 or 50 minutes at 250 v (40 to 60

[1] Label varnish, Cenco no. 11380.

[2] No. 72 vegetable parchment powder paper available through the Upjohn Co.

[3] Schleicher and Schuell filter paper, grade no. 900 cut into 3.5 × 4 inch rectangles.

ma), the agar plate was dried under an infrared lamp. The parchment paper, now having on its surface the dried, separated sample, was detached from the glass plate and stained for 20 to 30 minutes in 0.1 per cent bromphenol blue solution. Excess dye was removed by successive rinsing in a 5 per cent solution of acetic acid until the background became white. The stained paper was air dried and then exposed to ammonia fumes to intensify the color.

For the quantitative determination of the protein components, the individual electrophoretic bands were cut from the pattern and the bromphenol blue was eluted with a 2 per cent solution of sodium carbonate in 50 per cent methanol. After 30 minutes of occasional shaking the solutions were decanted and their optical densities were measured at 590 mμ using a Coleman spectrophotometer.

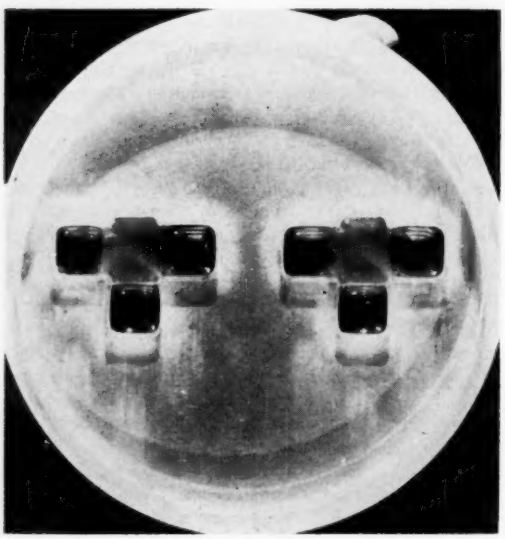
Immunological reactions. The Ouchterlony double diffusion technique as modified by Wilson and Goodman was used (16). This method is considered to be more sensitive than the one using the typical five basin Ouchterlony plate (17). The modified plates (3⅝ inches in diameter) were made of ¼ inch Lucite and contained two "T" shaped wells as shown in Figure 2A. These wells were filled with a 0.75 per cent solution of agar in a 13 per cent NaCl solution. After the gel had formed, the agar was removed from the three 10 × 10 mm basins, nos. 1, 2 and 3, thereby leaving the agar block no. 4 (Figure 2B). Wells 1 and 3 were reserved for the sample to be tested while no. 2 was used for the appropriate chicken antiserum. The precipitin reaction occurred in the agar block as a crescent extending from well 1 to well 3 as shown in Figure 2B.

In all experiments chicken antiserum (18) specific for human albumin and γ-globulins was used to develop the albumin and γ-globulins of the cervical mucus.

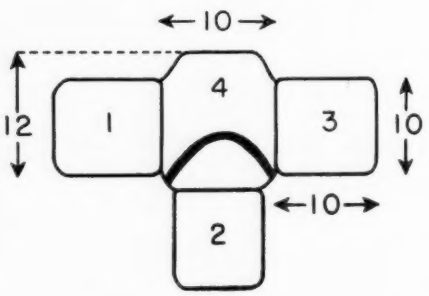
RESULTS AND DISCUSSION

The total protein of cervical mucus, determined on a pooled sample (10 cases in different phases of the menstrual cycle) with a biuret method (19), was found to be 1.1 per cent. This value confirmed that of Shettles and associates (3). It would have been desirable to determine the total proteins in individual samples. However, this was not feasible because of difficulties experienced in the quantitative sampling of the small amounts of each specimen available and because of the lack of an adequate micromethod.

Electrophoresis on agar-gel invariably resulted in a separation of proteins into four fractions (Figure 3). These were: *a*) a compact band migrating similarly to that of serum albumin; *b*) a diffuse band between the origin and the albumin zone; *c*) a "nonmigrating" fraction which remained at the origin and on the strip of filter paper used to apply the sample; and *d*) a band moving toward



A



B

FIG. 2. A. MODIFIED OUCHTERLONY PLATE OF WILSON AND GOODMAN (16, 17). B. DIAGRAM SHOWING PRECIPITIN REACTION IN AGAR BLOCK NO. 4. Wells 1 and 3 were used for samples while 2 was reserved for antiserum. Dimensions in the diagram are given in millimeters.

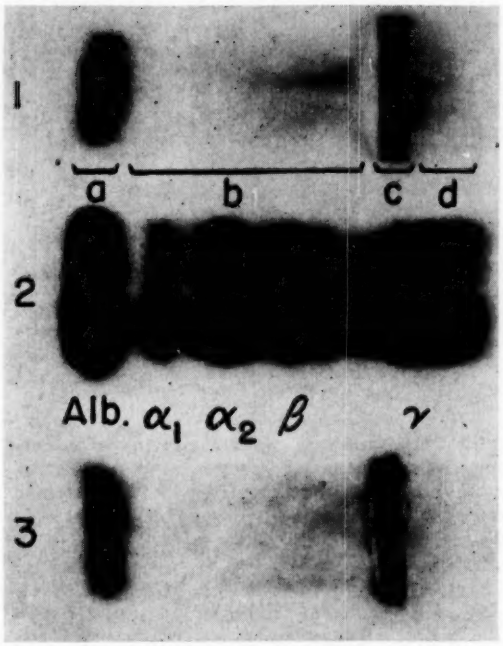


FIG. 3. THE ELECTROPHORETIC SEPARATION OF PROTEINS OF CERVICAL MUCUS IN AN AGAR GEL MEDIUM. 1 and 3 = cervical mucus; 2 = normal human serum.

the cathode and resembling γ-globulin. In some cases a fairly well defined band was observed similar to that of β-globulin.

Several samples were separated electrophoretically on a Mylar film in place of the usual parchment paper. After drying, these runs were stained by a PAS technique (20). All fractions except the albumin reacted positively. The nonmigrating portion exhibited the most intense reaction for carbohydrate suggesting that the mucopolysaccharide component did not migrate under the conditions of these experiments.

To determine the quantitative distribution of the protein, the electropherograms were divided into three areas: fraction a (albumin), combined fractions b and d (globulins), and fraction c (nonmigrating). The relative distribution of these components is shown in Table II. The 20 cases in this series were subdivided into the usual three periods: postmenstrual, the period dating from the completion of menstruation through the tenth day of the cycle; midcycle, from the eleventh through the eighteenth day of the cycle; and premenstrual, from the nineteenth day of the cycle to the onset of menses. The average percentage of each fraction was calculated for each period as well as for the complete cycle. The amount of albumin was about 21 per cent of the total proteins, while the total globulin fraction was 36 per cent, and the nonmigrating portion averaged 43 per cent. The data are suggestive of a cyclical change in the proportion of the proteins, with albumin and globulins tending to be somewhat reduced during midcycle. However, these variations are not statistically significant.

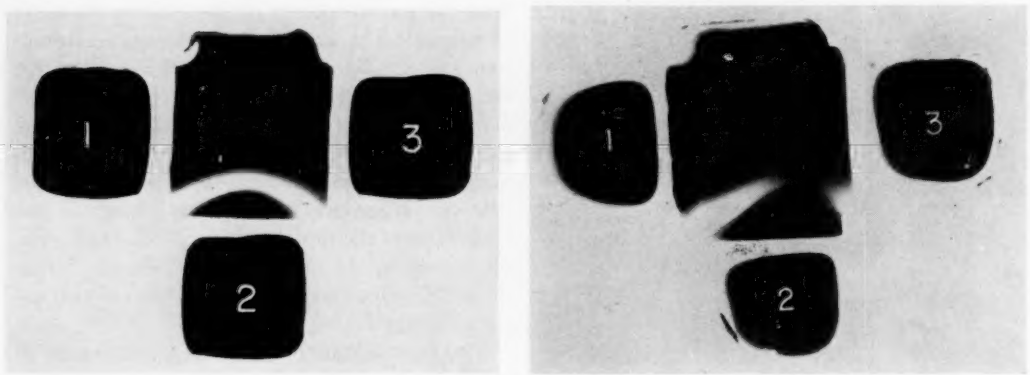
The identification of the protein components of cervical mucus cannot be based only on electrophoretic mobility. The presence of albumin and γ-globulin fractions was confirmed by an immunological reaction using the modified Ouchterlony plate of Wilson and Goodman (16, 17). The albumin and γ-globulin fractions of cervical mucus

TABLE II

Relative percentage of electrophoretically separated proteins from the cervical mucus

Case	Day of cycle*	Percentage of fractions		
		Albumin	Globulin	Nonmigrating
		Postmenstrual		
1	4	14.6	35.6	49.8
2	6	28.0	46.6	25.4
3	7	28.2	35.6	36.2
4	8	16.1	51.0	32.9
5	9	18.5	54.7	26.8
Average		21.1	44.6	34.2
		Midcycle		
6	12	5.0	20.5	74.5
7	13	12.1	30.5	57.5
8	13	5.2	20.3	74.4
9	14	44.1	27.6	28.3
10	15	12.6	46.7	40.7
11	15	22.5	36.2	41.2
Average		16.9	30.3	52.8
		Premenstrual		
12	19	25.3	36.8	37.9
13	20	25.5	30.7	43.8
14	21	10.3	29.4	60.3
15	21	20.9	37.2	41.9
16	23	12.2	23.7	64.0
17	24	32.5	50.0	17.5
18	25	14.9	23.4	61.7
19	28	32.7	38.9	28.6
20	30	37.9	35.7	26.4
Average		23.6	34.0	42.5
Total average for the cycle		20.7 ± 10.7	35.6 ± 10.2	43.5 ± 16.5

* Day of cycle on which the sample was collected.



A B

FIG. 4. IMMUNOLOGICAL REACTIONS USING WILSON MODIFIED OUCHTERLONY PLATES FOR PROTEINS OF HUMAN CERVICAL MUCUS. A. REACTIONS OF IDENTITY USING ELECTROPHORETICALLY SEPARATED ALBUMIN AFTER A DIFFUSION TIME OF 2 DAYS. 1 = Albumin from cervical mucus; 2 = immune anti-albumin antiserum; 3 = pooled human serum. B. PRECIPITIN REACTION USING ELECTROPHORETICALLY SEPARATED γ-GLOBULIN AFTER A DIFFUSION TIME OF 3 DAYS. 1 = γ-Globulin from cervical mucus; 2 = immune anti-γ-globulin antiserum; 3 = pooled human serum.

were first separated electrophoretically. Then the agar plate was frozen and the albumin and γ-globulin zones were cut out of the electropherogram using a concurrently run prestained sample of human serum to locate these regions. Corresponding bands from several separations were pooled. The γ-globulin mixture prepared in this manner was concentrated by dialysis against a 20 per cent solution of polyvinylpyrrolidone before use. Electrophoretically separated albumin and γ-globulins from cervical mucus and pooled human serum were tested, respectively, against immune chicken antihuman sera, specific for albumin and γ-globulins (18). In all cases a precipitin reaction developed within 72 hours (Figure 4).

The identification of other electrophoretically separated fractions of cervical mucus, namely, those associated with the alpha and beta regions, was not feasible with this technique. It appears, contrary to the opinion of some investigators, that cervical mucus normally contains both albumin and γ-globulin, which are immunologically identical with those of human serum. The nonmigrating and strongly PAS-positive protein fraction of the mucus may be associated with the mucopolysaccharides which are known to be present in cervical secretion. The absence of electrophoretic mobility may have been the result of adsorption to the filter paper.

SUMMARY

1. Electrophoresis in agar-buffer medium has been found to be a suitable technique for the study of the proteins of cervical mucus.

2. Albumin and γ-globulin were shown to be normally present in the cervical secretion of 20 women during various phases of the menstrual cycle. These proteins were identified by immunological techniques.

3. The total protein content of the pooled mucus was found to be about 1 per cent.

4. On the average, there were about 21 per cent albumin, 36 per cent globulins, and 43 per cent of a nonmigrating component in the cervical mucus. The latter fraction was probably associated with the mucopolysaccharides.

5. All of the electrophoretically separated proteins except albumin gave a positive periodic acid-Schiff reaction.

ACKNOWLEDGMENTS

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ENZYMATIC ACTIVITY OF THE ISOLATED GLOMERULUS IN NORMAL AND NEPHROTIC RATS *

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Recent studies of the nephrotic kidney have implicated the glomerulus as the primary anatomic site of injury. Using the electron microscope, Farquhar, Vernier and Good (1) and other investigators have demonstrated that the most consistent and striking alteration is a lesion in the foot process of the glomerular epithelial cell. Hjelt, Stjernvall and Hallman (2), in a study of congenital nephrosis, found imperfectly formed or absent foot processes. Vernier, Papenmaster and Good (3), and Harkin and Recant (4) have described similar lesions of the foot process in experimental nephrosis induced by the aminonucleoside of Puromycin, and Piel (5) has made similar observations in nephrosis produced by antikidney serum. In the experimentally induced lesion, glomerular injury is found prior to tubular changes (4).

With the recognition that relatively specific anatomic changes occur in the glomerulus in nephrosis, it seemed pertinent to determine if alterations occur in glomerular enzyme activities. The application of quantitative histochemical techniques seemed to be the most promising approach for such a study.

Previous histochemical studies of kidney utilizing staining techniques have been "qualitative" in nature in that they demonstrated the presence or absence of enzyme, but could not quantify these enzymes. In addition, such studies have given most attention to the renal tubule rather than to the glomerulus.

Quantitative histochemistry utilizing microchemical methods was first devised by Linderstrøm-Lang (6), and later modified and extended by Lowry and associates (7–10). These methods were applied to kidney investigation in 1956 by McCann (11), who made preliminary measurements of alkaline phosphatase, aldolase and fumarase activities in the glomerulus of the normal dog. Bonting, Pollak, Muehrcke and Kark measured the activities of alkaline phosphatase (12) and lactic dehydrogenase (13) in single glomeruli and in other parts of the nephron of various species.

This paper is concerned with a description of some of the enzymatic activities of the isolated glomerulus in normal rats and with the alterations which occur with the induction of aminonucleoside nephrosis. Utilizing quantitative histochemical methods, alkaline phosphatase (Alk. P'tase), hexokinase (HK), glucose-6-phosphate dehydrogenase (G-6-PDH), lactic dehydrogenase (LDH), malic dehydrogenase (MDH), and isocitric dehydrogenase (ICDH) activities were measured. These observations represent the initial step in an effort to characterize the biochemical lesion in the experimental nephrotic syndrome.

METHODS

Twelve male Sprague-Dawley rats with a starting weight of 74 ± 8 g were used and divided into an experimental and control group. Animals were housed in individual cages and pair-fed with Ralston-Purina rodent chow. Water was allowed *ad libitum*. Nephrosis was produced in one group by administration of daily subcutaneous injections (0.3 ml per 100 g weight) of a 0.5 per cent solution of aminonucleoside (6-dimethyl-aminopurine-3′-amino-D-ribose) (14, 15). Urine protein excretion was measured daily by the Esbach method (16). At various stages of the nephrotic syndrome, pairs of rats having an average weight of 105 g (one from each group) were sacrificed by decapitation following an overnight fast. Blood was collected in heparinized tubes for the determination of cholesterol (17). The kidneys were then rapidly dissected and handled as follows.

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† Fellow of the Foundation for Biol.-Med. Research (Switzerland). Supported by the American-Swiss Foundation for Scientific Exchange, Inc. Present address: Med. Universitaets Poliklinik, Basel, Switzerland.

1. Preparation of tissue. The methods and equipment used have been described in detail by Lowry (7, 8). Blocks of tissue (125 mm^3) from the upper and lower poles of the right kidney were rapidly excised with a chilled razor blade and plunged into liquid nitrogen. The upper half of the left kidney was used for dry weight determinations. The remaining kidney tissue was cut into small pieces, blotted, placed in liquid nitrogen and then crushed in a chilled mortar. The resulting tissue powder was lyophilized. The two pieces of the right kidney were mounted without thawing on 4 per cent tragacanth USP in 0.9 per cent NaCl solution and sectioned with a microtome in a cryostat at $-25°$ to $-30°$ C. Multiple sections of 28 to 30 μ thickness were cut from each block of tissue and were then removed to a glass container for lyophilization. Additional sections of 10 and 30 μ were obtained for staining and histological inspection.

The tissue powder and sections were simultaneously lyophilized at $-45°$ C and 0.001 mm mercury for 12 to 18 hours. The tubes were then brought to room temperature over a period of 2 hours. Sections and tissue powder from each pair of animals were redistributed into 6 lyophilization tubes, evacuated and stored at $-20°$ C until used. This made possible simultaneous analysis of control and experimental samples for each of the 6 enzymes studied without necessitating refreezing of material. In addition, an attempt was made to randomize sections from various portions of the tissue block so that enzyme analyses were not performed upon serial sections.

Problems concerning absorption of air and moisture by the sections, weighing of the samples and stability of enzymes during storage have been previously described by Lowry (8). In order to control the experiment further, the enzymatic activities were always studied in a fixed time sequence, namely, Alk. P'tase, one day after preparation of the tissue powder and section; LDH, two days after preparation, and so forth.

2. Tissue dissection. The lyophilization tubes were allowed to warm to room temperature before opening. With the aid of a hot towel this process required one-half hour. Air was then admitted to the containers, and the dried unstained sections were placed on the stage of a Spencer dissecting microscope and studied at a magnification of 40 to 90 ×. Individual glomeruli and vessels were easily visualized and were dissected manually (free-hand) using a sharp no. 26 gauge stainless steel needle inserted into the shaft of a tuberculin syringe. Care was taken not to cut but rather to tease out the structure from the surrounding area before dissection. All dissections of questionable cleanliness (i.e., contaminated with parts of surrounding structures) were discarded. Glomeruli were chosen from at least two sections and from different parts of these sections, in order to obtain a better randomization of the material. In addition, arterioles from cortex and medulla (diameter smaller than

TABLE I

Salient details of enzyme determinations

Enzyme*	Buffer†	Substrate	Cofactors	Dry weight of dissected tissue	Incubation volume	Incubation time	End-products of reaction
				μg	μl	*min*	
Alk. P'tase (9)	0.5 M AMP_1‡ pH 10.0	8 mM Disodium-*p*-nitrophenyl phosphate	2 mM Mg	0.02–0.20	10.0	60	*p*-Nitrophenol
G-6-PDH (18)	0.1 M AMP_2§ pH 9.3‖	5 mM Glucose-6-phosphate	0.3 mM TPN	0.03–0.11	8.8	60	TPNH Gluconate-6-phosphate
HK (18)	0.05 M Tris pH 8.1	8 mM Anhydrous glucose	0.3 mM TPN 3.0 mM ATP 2.4 mM G-6-PDH (crude)¶	0.80–3.5	22.0	60	TPNH Gluconate-6-phosphate
LDH (10)	0.1 M Tris pH 7.4	1.5 mM Na pyruvate	1 mM DPNH	0.03–0.18	20.0	30	DPN lactate
MDH (19)	0.1 M AMP_1‡ pH 10.5	120 mM Malate	4.5 mM DPN	0.02–0.12	22.0	30	DPNH oxalacetate
ICDH (20)	0.1 M Tris pH 8.1	5 mM Isocitrate	1 mM TPN	0.04–0.24	10.0	60	TPNH α-Ketoglutarate

* Numbers in parentheses are reference numbers.

† All buffer substrates contained 0.05 per cent bovine plasma albumin to increase stability of enzyme during incubation.

‡ AMP_1 = 2 amino-2 methyl-1 propanol (Eastman Kodak).

§ AMP_2 = 2 amino-2 methyl-3 propanediol (Eastman Kodak).

‖ At pH 9.3, 5 to 10 per cent of the activity measured is due to 6-phosphogluconic dehydrogenase in the tissue.

¶ Prepared in Dr. Lowry's laboratory from pig adrenal containing 0.2 per cent HK.

100 μ) were dissected. The weight for all specimens ranged between 0.015 and 3.5 μg. Glomerular samples were 0.015 to 0.110 μg.

3. Analytical methods. The dissected glomeruli and arterioles were weighed individually on two "fish-pole" quartz fiber balances (useful range from 0.010 to 0.120 μg and 0.5 to 5.0 μg), as described by Lowry (7), and were then placed into small test tubes with an inner diameter of 2 or 4 mm. This was performed with the aid of a wheeling device on a mechanical stage under the microscope (8). Low activity for certain enzymes required pooling of several samples in one tube.

Ten to 20 mg of the dry tissue powder material was gently homogenized by hand with a glass homogenizer in a 1:10 dilution in a chilled buffer (Table I). The homogenate was allowed to sit for 45 minutes or more before further dilution and final chemical analysis. Enzymatic analyses of tissue powder were performed simultaneously with analyses of the sections which were processed immediately. Homogenate activity was determined in triplicate and in two different dilutions varying with the enzyme tested. Glomerular enzyme analyses varied from 5 to 53 determinations for each enzyme studied.

Enzyme determinations. The methods employed were devised by Lowry and associates and are summarized, as adapted to the study of the kidney, in Table I. Coenzyme changes, measured in a model A Farrand fluorimeter, formed the basis of most activity measurements (TPNH, DPN, DPNH) (10). Alk. P'tase activity was measured as *p*-nitrophenol formation in a Beckman DU spectrophotometer. Enzyme activity was expressed as moles of substrate transformed per kilogram of dry weight of tissue per hour. In addition to the dry weight as a standard of reference for enzyme activities, protein (21) and deoxyribonucleic phosphate (DNA-P) (22) determinations were carried out on glomeruli and homogenates. This was deemed essential in view of the possible alterations in tissue protein content in nephrosis.

Initially, tissue blanks were run for both homogenates and sections. These were later omitted since they were found not to be different from the substrate-reagent blanks. In order to rule out fluorescent interference from aminonucleoside, a 0.005 per cent solution in redistilled water was tested. No reading, compared with a dilute quinine sulfate solution of 0.05 μg per ml, was noted. There was also no fluorescence noted when the drug was added to homogenates.

RESULTS

Animals treated with aminonucleoside developed proteinuria of varying degree (75 to 216 mg per 24 hours). Plasma cholesterol levels in the nephrotic animals ranged from 348 to 529 mg per 100 ml, while the control levels were 76 to 100 mg per 100 ml. Ascites, when present in the nephrotic group, paralleled the severity of the proteinuria. Gross and microscopic examinations of the kidneys in this syndrome have been previously reported (14, 15).

TABLE II

Rat kidney enzyme activity

Enzyme*	Isolated glomeruli Normal	Isolated glomeruli Nephrotic	p†
Alk. P'tase	3.07 ± 0.33‡	2.24 ± 0.30	<0.01
HK	0.534 ± 0.028	0.690 ± 0.078	NS
G-6-PDH	1.190 ± 0.98	1.600 ± 0.084	<0.005
LDH	26.6 ± 3.0	32.2 ± 2.4	<0.05
MDH	32.0 ± 1.7	40.3 ± 2.5	<0.01
ICDH	4.92 ± 0.49	5.59 ± 0.61	NS
	Homogenates		
Alk. P'tase	25.03 ± 4.37	13.35 ± 2.28	<0.05
HK	0.976 ± 0.067	1.202 ± 0.095	<0.01
G-6-PDH	1.210 ± 0.064	1.760 ± 0.228	<0.05
LDH	68.9 ± 5.5	69.0 ± 5.2	NS
MDH	89.1 ± 2.5	80.9 ± 4.7	NS
ICDH	33.00 ± 1.4	26.50 ± 3.5	NS

* Activity expressed as moles per kg dry weight per hour.
† Probability based on the means of both groups (23).
‡ Mean ± SEM (6 animals).

A. Analyses of isolated glomeruli and whole kidney homogenates

Enzymatic analyses were made on single glomeruli whenever possible or on pools of 2 to 7 glomeruli. From 2 to 15 determinations were carried out to arrive at a mean value per enzyme per animal. Similar analyses were made on homogenates. Table II summarizes the mean and standard error of the mean (SEM) for all enzymes tested in the control and in the nephrotic animals. The statistical method employed was that of the analysis of variance for paired data (23).

Protein determinations per unit of dry weight were performed on 20 to 30 glomeruli. Glomeruli from each animal in the two groups were pooled for each determination. No significant difference was noted between the normal (663 ± 19 g protein per kg dry weight) and the nephrotic group (661 ± 10 g protein per kg dry weight). Protein determinations on whole kidney homogenates also revealed no significant differences. The mean values were 613 ± 28 g per kg dry weight in the normals and 632 ± 42 g per kg in the nephrotics. Spot assays of DNA-P[1] content of pooled glomeruli revealed values of 82.0 mmoles per kg dry weight in one nephrotic and 90.7 mmoles per kg in the pair-fed control animal. The lack of significant

[1] We are grateful to Dr. G. Morrison who carried out the determinations of DNA-P.

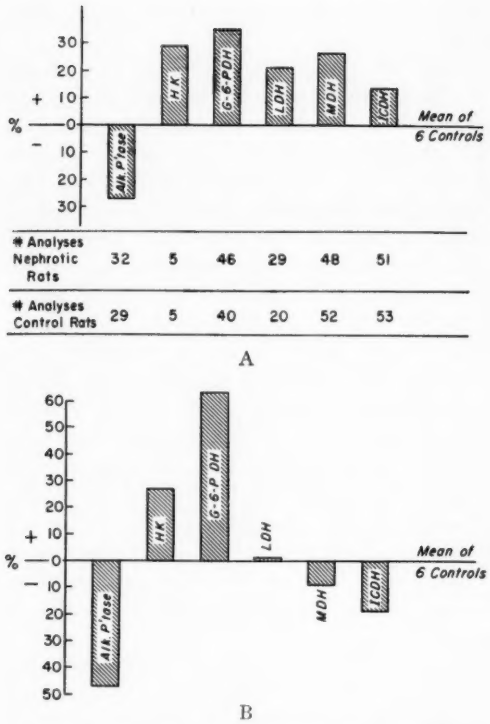

FIG. 1. A. PER CENT CHANGE IN GLOMERULAR ENZYME ACTIVITY IN NEPHROTIC RATS COMPARED WITH CONTROLS. B. PER CENT CHANGE IN KIDNEY HOMOGENATE ACTIVITY IN NEPHROTIC RATS COMPARED WITH CONTROLS. Homogenate refers to lyophilized tissue powder of the whole kidney. Each bar represents two dilutions of homogenate in triplicate for six nephrotic rats.

differences between the protein or DNA-P content per unit of dry weight of glomeruli or homogenate in normal and nephrotic animals permits comparison of enzymatic activity on a dry weight basis.

Comparison of normal and nephrotic kidney enzymes may be seen in Figures 1A and 1B, which summarize the over-all enzymatic changes in glomeruli and homogenates as a result of the induction of the nephrotic state. The most striking change in both glomeruli and homogenates may be seen in the activities of Alk. P'tase and G-6-PDH. The former showed a significant decrease in activity, the latter a marked increase. LDH, MDH and HK tended to be increased in glomeruli, while in homogenates only the HK increased. The other enzymes showed no significant change.

An attempt was made to assess the effect of proteinuria upon the development of the enzymatic changes. Nephrotic animals were divided into three groups, two animals in each, on the basis of the magnitude of proteinuria. Figures 2A and 2B plot, respectively, the percentage change in glomerular and homogenate enzymes as related to the level of proteinuria and compared with the

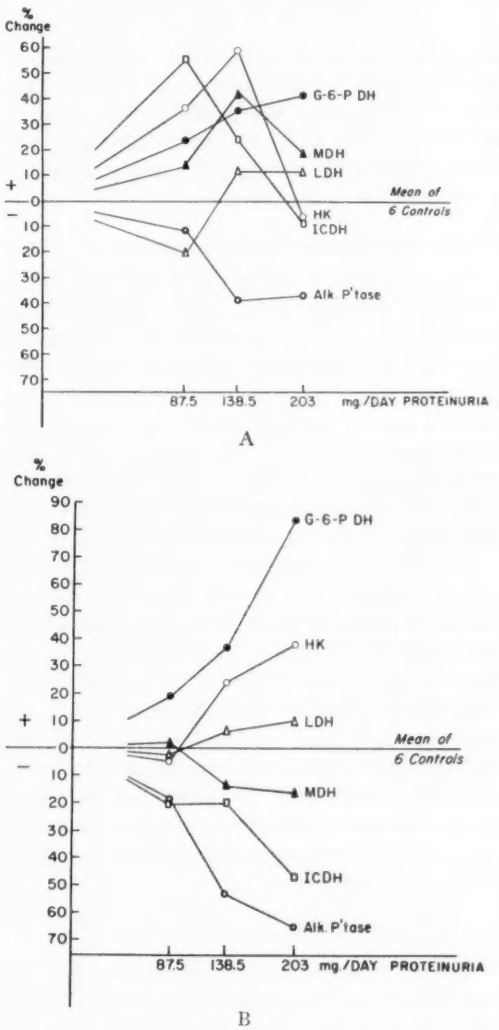

FIG. 2. A. GLOMERULAR ENZYME ACTIVITY CHANGES WITH PROGRESSIVE PROTEINURIA. B. KIDNEY HOMOGENATE ENZYME ACTIVITY CHANGES WITH PROGRESSIVE PROTEINURIA. Each point represents multiple determinations on two animals in both A and B.

TABLE III

Mean enzyme activities of isolated rat kidney arterioles

Enzyme*	Control†	Nephrotic
Alk. P'tase	19.62 [4]	14.03 [5]
G-6-PDH	0.845 [3]	1.099 [5]
LDH	33.3 [4]	30.6 [6]
MDH	30.4 [5]	32.7 [4]
ICDH	5.66 [5]	4.11 [4]

* Activity expressed as moles per kg dry weight per hour.
† Number of determination is given in brackets.

entire group of six normal animals. It is apparent that the pattern of glomerular change differed from that in homogenate. Glomeruli showed an early increase in many activities with a subsequent decrease, whereas the homogenate changes appeared to be consistent in one direction.

B. Miscellaneous enzymatic analyses

The main constituent of the glomerulus is the capillary. Although the anatomic composition of arterioles is more complex than that of capillaries, both have a comparable embryonic origin. The possibility that similar biochemical changes might occur in both of these structures made desirable a comparison of enzymatic activity in the normal and nephrotic kidney arterioles. The mean enzyme activity of a number of single kidney arterioles from both groups of animals is charted in Table III.

The influence of red blood cells in glomeruli on enzyme activity was studied in normal kidney. Bloodless glomeruli in the unstained sections had an average ICDH activity of 7.3 moles per kg per hour, compared with 5.4 moles per kg per hour in blood-filled glomeruli. This difference in activity, however, could have had little influence on the final results reported here, since all glomeruli were selected at random for dissection and no microscopic evidence of differences in blood content of nephrotic glomeruli when compared with normals could be demonstrated.

DISCUSSION

Despite intensive investigation, there are few established facts concerning the intrinsic metabolism of the kidney glomerulus. One of the obstacles in the path of classical biochemical study of the glomerulus has been the small amount of tissue which is available for study. This void of direct biochemical information has been partially filled by the results of histochemical staining procedures.

In the rat glomerulus, Wachstein and Meisel (24) have demonstrated the presence of glucose-6-phosphatase, esterase (alpha-napthyl-acetate), TPN diaphorase and DPN diaphorase enzyme activities. They were unable to find acid or Alk. P'tase, glycerophosphatase, 5-nucleotidase, glucuronidase or succinic dehydrogenase. One of the problems encountered in the interpretation of staining techniques results from the "all or none" effect, in that low activities may be missed. This is most clearly evident in the studies of glomerular Alk. P'tase activity. Whereas Wachstein and Weisel obtained negative results, McCann (11), Bonting and co-workers (12), and the present authors have been able to demonstrate Alk. P'tase activity in glomeruli by quantitative biochemical techniques. Although staining procedures permit localization of enzymes, it appears that the direct quantitative methods used here allow histological localization as well as detection of small changes in activity.

The choice of the six enzymes studied was determined by the effort to sample anaerobic glycolytic enzymes (HK and LDH), the hexose-monophosphate shunt with G-6-PDH, as well as enzymes of the oxidative pathways (MDH and ICDH). The addition of Alk. P'tase, particularly in the analysis of whole kidney homogenate, seemed desirable for purposes of comparison, in view of its common usage in many staining studies of renal tubules. Its over-all metabolic importance, however, is poorly understood at the present time.

We are aware of the fact that the results obtained for the six control animals do not necessarily represent normal enzymatic activities. The pair feeding of the control animals with the nephrotic group resulted in a small limitation in food intake in the controls, and the effect of this factor has not been separately studied. With this reservation in mind, the results of our studies may be discussed:

Normal rat glomeruli. On examination of the enzymatic activities in the normal rat glomerulus (Table II), one is impressed by the high levels of activity for the oxidative enzymes (MDH and ICDH) and for LDH, which are in contrast with the levels of HK and G-6-PDH. Alk. P'tase is only moderately active when compared with the above noted enzymes. The levels of Alk. P'tase

and LDH are of the same order of magnitude as those reported by Bonting and associates. It is, unfortunately, impossible to compare our data with results of studies of glomerular enzymes obtained by staining techniques.

Normal rat kidney homogenate. A distribution of enzymes similar to that in glomeruli is also found in the normal rat kidney homogenate. However, the levels of activity in homogenates are significantly higher than those in glomeruli, with the exception of G-6-PDH which is the same in both glomeruli and homogenates. The ratios of activity in homogenate relative to glomeruli are 8.1 for Alk. P'tase, 1.8 for HK, 2.6 for LDH, 2.8 for MDH, and 6.7 for ICDH. The low ICDH activity in glomeruli suggests a relatively unimportant role of the Krebs cycle in the glomerulus, with the reservations inherent in all artificial assay systems. The highest ratio is observed for Alk. P'tase. It is known that the renal tubules, which are the major components of the homogenate, have higher activities for these enzymes (11, 13).

Since quantitative data on the enzymes of liver (25) and brain (26) are available, it seemed of interest to compare these activities with those in kidney (Table IV). The most striking difference may be seen in the Alk. P'tase activity. Kidney homogenates surpass by far the activity found in the two other organs. G-6-PDH shows essentially the same level of activity in all three. The oxidative enzymes (MDH and ICDH) and LDH in kidney and liver are similar. HK is more active in brain than in kidney and in liver. The interpretation of these data is somewhat limited by the fact that observations were not made upon organs obtained from the same animal species in all cases, although it is clear that differences of the order noted for Alk. P'tase are nevertheless significant.

Effect of nephrosis on kidney enzymes. With the induction of nephrosis, definite changes in enzymatic activity are noted in glomeruli and kidney homogenates (Figures 1A and 1B). Alk. P'tase activity is strikingly reduced in glomeruli and homogenates, while G-6-PDH activity is increased in both. Although HK activity is also increased in both glomeruli and homogenates, the p value proved not to be significant for glomeruli, but it must be noted that only a small number of determinations was made. Significant enzyme activity increases for LDH and MDH were obtained in glomeruli only, whereas for the two enzymes in homogenates, no change was found. ICDH showed no significant change in either.

Fisher and Gruhn, using staining techniques, studied activity changes in nephrotoxic (28) and aminonucleoside (29) nephrotic rats. In both types of nephrosis they found no alterations in lipase, esterase, glucose-6-phosphatase, acid and Alk. P'tase, and 5-nucleotidase activities of renal cortex and medulla. However, succinic dehydrogenase and cytochrome oxidase showed decreased activities. Wachstein and Lange (30), in their histochemical study of nephrotoxic nephritis in the rabbit, in contrast to the findings of Fisher and Gruhn, observed a decrease in Alk. P'tase activity of kidney tubules. This was the earliest enzymatic alteration noted. In addition, glycerophosphatase and 5-nucleotidase were noted to be less active, as

TABLE IV

Enzyme activity of brain, liver and kidney

Organ	Enzyme					
	Alk. P'tase	HK	G-6-PDH	LDH	MDH	ICDH
Rabbit brain*: Homogenate	0.60	10.0	1.80	70	80	11.2
Rat liver†:		0.30‡				
Portal area	0.38		1.02	137.7	73.1	10.3
Central area	0.19		0.77	76.3	67.7	12.3
Rat kidney†: Homogenate	25.03	0.976	1.21	68.9	89.1	33.0

* Moles per kg *lipid-free* dry weight per hour. One-half the values recorded correspond approximately to moles per kg dry weight per hour (26).

† Moles per kg dry weight per hour.

‡ Whole rat liver homogenate (27).

were other enzymes including those studied by Fisher and Gruhn.

In general, depression of histochemical enzymatic activity paralleled the severity of renal disease. No comments were made about glomerular enzyme activities. Our findings indicate a definite change in glomeruli as well as in homogenates.

If the enzymatic changes noted are related to proteinuria, then one might anticipate an intensification of the changes with time and increased proteinuria. In an effort to answer this question, the data were plotted as noted in Figures 2A and 2B. The interpretation of the small number of data and the arbitrary grouping must be approached with caution. However, it would appear that an intensification of the changes with increased proteinuria does occur in both glomeruli and homogenates for the Alk. P'tase and G-6-PDH enzymes. Further, it is apparent that certain differences in the enzymatic activity patterns in glomeruli and homogenates exist. Whereas some of the enzymatic changes in glomeruli are characterized by early rises with later declines in activity, this is not true for homogenates.

Neither an absolute biochemical nor a morphologic interpretation of this behavior is possible. It is obvious that the enzymatic activity of the minute total glomerular mass is drowned in the preponderance of renal tubules representing the homogenate. The early changes observed in glomeruli which differ from homogenates might point to specific enzyme alteration, possibly related to the podocyte lesions observed by electron microscopy. The later changes in both glomeruli and homogenates may well be secondary ones, associated with continued proteinuria, or with a generalized type of tissue response to injury. This type of general response is made more likely in that the enzymatic changes in renal arterioles (Table III) are in the same direction as those of glomeruli and also homogenates.

These considerations raise the following questions which are at present under investigation in our laboratory: *1*) Can one obtain more definitive evidence of the specificity of glomerular changes by studies conducted prior to development of proteinuria? *2*) Do similar enzymatic changes occur in the glomeruli and tubules with other types of nephrosis or other renal lesions leading to proteinuria? *3*) Do other organs, despite normal histological appearance, share in these enzymatic changes?

These questions arise by virtue of the fact that although the concentrations and distribution of enzymes appear to be characteristic of various structures and organs, nevertheless, it is possible that a variety of injuries may result in the same pattern of enzymatic response. Finally, it is possible that the enzymatic alterations described are secondary or adaptive in nature and represent a response to the "antimetabolic" action of the aminonucleoside.

It has been shown that the glomerulus is an active metabolic unit with considerable enzymatic activity. It is tempting to speculate that the relatively high concentration of G-6-PDH in the glomerulus compared with the glomerular/tubular ratios of the other enzymes relates in some way to its basic functions.

SUMMARY

1. Quantitative histochemical methods have been adapted for the study of six enzymes (alkaline phosphatase, hexokinase, glucose-6-phosphate dehydrogenase, lactic dehydrogenase, malic dehydrogenase and isocitric dehydrogenase) in isolated rat glomerulus, whole kidney homogenate and renal arterioles in normal and nephrotic rats. Enzymatic activity was expressed per unit of dry weight. Protein levels per unit of dry weight did not differ in the two groups.

2. The normal rat glomerulus is a potentially active metabolic unit. With the exception of glucose-6-phosphate dehydrogenase, all enzyme activity levels are lower than those in kidney homogenates. Renal arteriolar enzyme activity is similar to glomerular activity with the exception of alkaline phosphatase, which is of the same magnitude as it is in homogenates.

3. The production of acute nephrosis with the aminonucleoside of Puromycin results in a significant increase in glucose-6-phosphate dehydrogenase, lactic dehydrogenase and malic dehydrogenase, and a marked decrease in alkaline phosphatase of glomeruli. The kidney homogenate activity of the nephrotic animals is significantly increased for hexokinase and glucose-6-phosphate dehydrogenase; a decrease is observed in alkaline phosphatase. These changes seem to be closely related to the severity of the disease as judged by proteinuria.

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QUANTITATIVE HISTOCHEMISTRY OF THE NEPHRON. II. ALKALINE PHOSPHATASE ACTIVITY IN MAN AND OTHER SPECIES *

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The classical experiments of Richards and his collaborators (1), which were started in 1920, laid the foundations for our understanding of renal function in terms of nephron anatomy. By direct puncture of renal tubules they demonstrated accurately the sites at which a variety of substances is absorbed or secreted by the nephron. This technique, which is continuing to provide useful knowledge in the hands of investigators such as Gottschalk and Mylle (2), and Wirz (3), cannot provide exact data on the enzymatic and other cellular mechanisms involved in the processes of tubular reabsorption and secretion. Detailed knowledge of the activities of specific enzymes in the individual anatomical and functional units of the nephron is necessary in order to understand these mechanisms, i.e., a knowledge of the "enzyme topography of the nephron."

Although conventional biochemical techniques using kidney slices or homogenates provide data on the enzyme activity of the kidney as a whole, they do not yield information of specific physiological importance because the individual functional units of the nephron cannot be studied by these techniques. Histochemical staining techniques are useful for purposes of orientation but do not yield quantitative results. In addition, when staining techniques are used to study enzyme distribution, the possibility always exists that artifacts might be caused by diffusion and absorption.

For these reasons the quantitative ultra-microchemical techniques of dissection, weighing, and assay of microscopically small fragments of tissue developed by Lowry and co-workers (4–6) for the brain, were adapted for study of the kidney (7, 8). In the present paper, the alkaline phosphatase distribution in the nephron of adult man, monkey, dog, rat, rabbit, frog, and toadfish is reported.

METHODS

The methods employed will be described only insofar as they differ from those developed by Lowry and associates (4–6). Slices of renal tissue from healthy animals were obtained as rapidly as possible after death. Human renal tissue was obtained by percutaneous renal biopsy (9) in four cases, and at autopsy in two (within 3 hours of death). The human tissue was obtained from persons with no clinical or histological evidence of renal disease.

Preparation of tissue for dissection. Immediately upon removal from the body the tissue was placed on a thin layer of a 5.0 per cent (weight/volume) tragacanth gel in 0.85 per cent NaCl [500 mg Mysteclin (Squibb) added per 500 ml to prevent fungal growth], which covered the top of a microtome sample holder (International Equipment Co., no. 3010). It was then immersed in liquid nitrogen and frozen rapidly.

Serial sections, 16 μ thick, were cut on a Minot rotary microtome (International Equipment Co., no. 3007 with safety razor holder no. 3017) in a cryostat (Harris Refrigeration Company, Cambridge, Mass.) at $-20°$ C. The first and third sections of every three cut were placed in a numbered well of a section holder (Acme Model Works, Chicago, Ill). The section holder containing the slices of tissue was inserted into a lyophilizing tube, and the sections were frozen-dried overnight in the cryostat at $-20°$ C. The evacuated tube was stored in a deep-freeze at $-35°$ C. The sections for enzyme assay were removed from the evacuated tube in the cryostat after temperature equilibration to $-20°$ C. They were placed in a second tube containing Drierite and brought to room temperature.

The middle section of every group of three cut was placed on a clean microscope slide, taken out of the cryostat, and allowed to thaw by warming the underside of the slide with the finger. The section was dried for 15 minutes in room air, immediately fixed for 5 minutes in 80 per cent ethyl alcohol, and stained by the periodic

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acid-Schiff (PAS) technique (5 minutes in 1 per cent HIO_4 in H_2O; rinsed in distilled H_2O; 10 minutes in 1 per cent fuchsin-1.9 per cent $Na_2S_2O_5$ in 0.5 N HCl; rinsed twice in 0.6 per cent $NaHSO_3$ in 0.05 N HCl; finally rinsed in H_2O). It was then counterstained with hematoxylin (5 seconds in hematoxylin-Harris, containing 4 volumes per cent CH_3COOH), rinsed for 30 seconds in tap water, passed through ethanol and xylene, and mounted in Permount.

Dissection of the frozen-dried tissue sections. The dissecting, weighing and insertion of tissue fragments into the substrate were all done in an air-conditioned laboratory maintained at a temperature of $23 \pm 2°$ C and a relative humidity of less than 40 per cent, in order to prevent condensation of moisture on the specimens.

The stained section was studied under a Galileo LGT/2 microscope with projection viewer (Sanders Laboratories, E. Rutherford, N. J.) and a map was drawn of the whole section, glomeruli and blood vessels serving as landmarks. The various anatomical parts of the kidney were identified (see below). When this was done, one of the two adjacent frozen-dried sections was placed on the stage of an A. O. Spencer Cycloptic dissecting microscope (type 53M-D2) and orientated with respect to the image of the stained section seen on the projection viewer. Each of the anatomical units of the kidney which had been identified in the stained section was then located in the adjacent lyophilized section, and each unit was dissected out freehand with microscalpels. From 5 to 10 specimens of each specific unit of the nephron were dissected out in this manner and their location in the section was recorded on the map.

TABLE I

Properties of alkaline phosphatase in the human kidney

Michaelis-Menten constant	0.35 mM
Optimal substrate concentration	5.0 mM
Optimal pH	10.0
Activity vs incubation time (37° C)	Linear 0 to 60 minutes
Activity vs enzyme concentration	Linear between 0.0 and 0.4 m moles/hour/L of incubation mixture
$V_{2°\,c}/V_{37°\,c}$*	0.17
Effect of 2 mM Mg^{++}	23% Increase at 37° C
Effect of 10 mM CN^-	99.4% Inhibition at 37° C

* V = velocity of the enzyme reaction at the temperatures given.

Weighing of tissue fragments. Each of the dissected specimens was transferred from the dissection microscope to a specimen holder by means of a hair point (4). The specimen holder was a chemically clean 10×25 mm glass

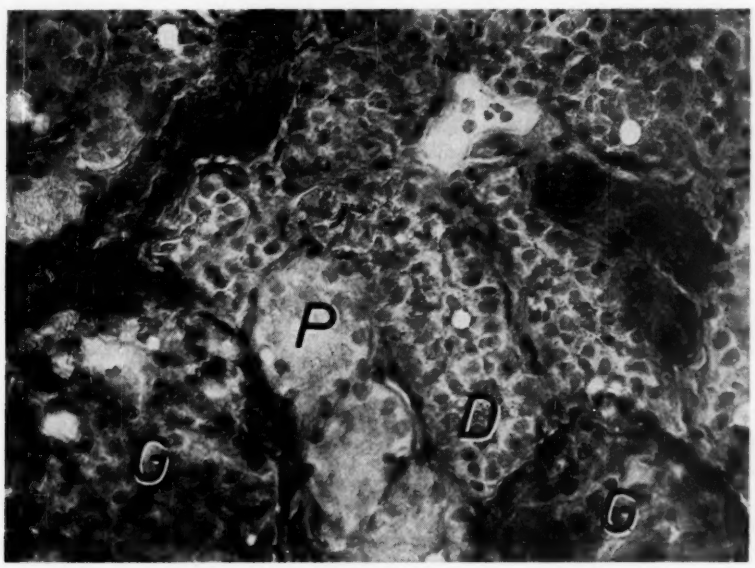

FIG. 1. SIXTEEN μ FROZEN SECTION OF RENAL CORTEX STAINED BY THE PERIODIC ACID–SCHIFF (*PAS*) TECHNIQUE AND COUNTERSTAINED WITH HEMATOXYLIN. Portions of two glomeruli are seen below (G). The proximal convoluted tubules (P) are broad and contain comparatively few nuclei in cross section. The distal convoluted tubules (D) are narrower, and many more nuclei are seen on cross section. Note the granules stained with PAS in the lumen.

slide, mounted on one end of a brass block 65 mm long × 12 mm wide × 10 mm high. Before weighing, the specimen holder, containing the fragments of tissue, was mounted on a manipulator, which was made from the mechanical stage of a microscope. The manipulator was used to load and unload the balance pan, and was placed on the left side of a horizontal stereoscopic microscope (A. O. Spencer, type 27K) which was used to read the displacement of the pan. This microscope was fitted with a vertical fine adjustment to facilitate the setting of the zero point.

The specimens were then weighed on a quartz fiber "fishpole" balance (4), with a sensitivity and reproducibility of a 0.4 mμg and a useful range of 10 to 100 mμg. The balance was calibrated with quinine hydrobromide crystals and the displacement of the quartz fiber was found to be related linearly to the sample weight over the entire useful range (10).

Enzyme assay technique. Each weighed specimen was inserted into 3 μl of substrate reagent contained in a microtest tube (ID 3 mm). This was done under the dissection microscope using a fine glass needle. Assays to determine alkaline phosphatase activity were then made using 3 μl of substrate reagent containing 0.5 M 2-amino-2-methylpropanol-1 (pH 10.0), 8 mM *p*-nitrophenylphosphate (Sigma), 2 mM $MgCl_2$, and 0.05 per cent bovine serum albumin (Armour). The conditions used for the assay were those we have found to be optimal for the human kidney (Table I). Omission of the 2 mM Mg^{++} from the assay medium decreased the enzyme activity by 19 per cent. Addition of 10 mM CN^- to the assay medium inhibited activity by 99.4 per cent.

After incubation for one hour at 37° C, the reaction was stopped and color was developed by adding 50 μl of 0.1 N NaOH. The optical density of the solution was read in Lowry-Bessey microcuvets at 410 mμ in the Beckman DU spectrophotometer. In each experiment suitable reagent blanks and standards were included. Tissue blanks were included in the initial experiments, but they were omitted subsequently after it had been found that the tissue, in the amounts used, did not increase the resulting optical density. The results were expressed in moles of substrate split per kilogram dry weight per hour (MKH units).

RESULTS

A. Morphology (Figure 1). To ensure that the individual anatomical and functional units of the nephron were dissected out accurately prior to chemical analysis, a detailed study of the morphology of the nephron was made both in stained and in unstained frozen-dried sections. As a result of these morphologic studies we were able to dissect out separately glomeruli, single proximal convolutions or single distal convolutions of the tubules, medullary rays, tubules from the outer and inner medullary zones, and collecting tubules from the base and apex of the papilla. In a few instances pars recta of proximal tubules, descending and ascending loops of Henle, and collecting tubules were separated out from the medullary rays and from the outer and inner zone of the medulla.[1] The various structures were identified and dissected out as follows.

The *glomeruli* were easily identified and the glomerular tufts dissected out free of Bowman's capsule. In the rabbit they were small and fragile. In the frog they occurred in clusters and appeared very light gray in the dry section where they were identified more easily than in the stained section.

The *proximal convolutions of the tubules* were broad, contained few nuclei in cross section and were composed of large cuboidal cells, which stained lightly with PAS (Figure 1). A distinct "brush border" was seen in the stained sections. In the dry sections they appeared gray and had no distinct lumen except in the rat, where the lumen was clearly visible. They formed the majority of the cortical tubules.

The *distal convolutions of the tubules* were usually narrower than the proximal, and contained many more nuclei on cross section (Figure 1). Some were identified by their intimate relationship to the macula densa. The cells were flat and the cytoplasm stained very lightly with PAS. PAS-stained granules of a uniform size were seen in the lumen in all species studied except the dog. In the dry sections the distal convolutions usually appeared darker than the proximal tubules, and a distinct lumen was visible.

The *medullary rays,* in all species but the frog and the toadfish, were composed of the straight

[1] The terminology used in this paper to describe the various zones of the medulla compares as follows with that of Oliver and associates (11):

Present authors		Oliver and associates (11)		
Medulla	Outer medullary zone	Outer stripe	Outer zone	Medulla
	Inner medullary zone	Inner stripe		
	Base of papilla	Inner zone		
	Apex of papilla			

part of the proximal tubules (pars recta), descending and ascending loops of Henle, and collecting tubules. In the stained sections the morphology of the pars recta was similar to that of the convoluted portion of the proximal tubules. The ascending limbs of the loops of Henle resembled the distal convoluted tubules. The collecting tubules were broad and contained many nuclei which appeared in two layers. Their cells were columnar, stained lightly with PAS, and the lumen contained many granules stained with PAS. The descending limbs of the loops of Henle were small round tubules with flat cuboidal cells.

In the unstained lyophilized sections the medullary ray contained two types of tubules, gray and dark tubules. The dark tubules were identified as the ascending limb of the loop of Henle. Two types of gray tubules were distinguished in the rabbit—one a dark gray, the other a light gray. It was difficult to identify them with certainty.

The *outer medullary zone* [outer stripe of outer medullary zone of Oliver and colleagues (11)], was composed mainly of pars recta, loop of Henle, and collecting tubules. The brush border of the pars recta of the rat stained more densely with PAS than did the convoluted portions of the proximal tubules.

The *inner medullary zone* (inner stripe of the outer medullary zone of Oliver and associates), contained collecting tubules and loops of Henle. The tubules were straight and converging toward the papilla. The loop of Henle appeared to curve around at the base of the papilla and return toward the outer medullary zone. In the dry section the collecting tubules were light and broad, whereas the loops of Henle were dark and narrow.

The *papilla* (inner medullary zone of Oliver and associates) was divided into a base and an apex, both consisting of collecting tubules, which were broad and composed of tall cuboidal cells which contained many nuclei. Many granules stained with PAS were seen in their lumina. In the dry section the papilla appeared uniformly light gray from base to apex. In the base there was a preponderance of longitudinal sections, while in the apex there was a considerable number of cross sections of tubules.

Convoluted tubules and collecting ducts were the only tubular structures recognizable in the kidney of the aglomerular toadfish, and there were large amounts of lymphoid tissue between the tubules. Proximal and distal convolutions could not be distinguished one from the other.

B. Alkaline phosphatase activity. The levels of alkaline phosphatase activity in the individual units of the nephron of healthy man, monkey (*Macacca mulatta*), dog (mongrel), rat (Sprague-Dawley), rabbit (New Zealand white), frog (*Rana pipiens*), and aglomerular toadfish (*Opsanus tau*) are given in Table II. This table also gives the number of individuals or animals studied and the number of fragments analyzed, together with the mean values obtained and the standard error of the means. The quantitative distributions are shown diagramatically in Figure 2. It should be noted that the alkaline phosphatase activity in the kidney of those species studied (man and dog) was inhibited completely by cyanide, whereas in human serum, liver and bone, appreciable amounts of noncyanide-sensitive alkaline phosphatase activity were found.

There were striking differences in the distribution and activity of alkaline phosphatase from one species to another. The highest levels of activity were found in the rat. The levels of activity were low in the nephrons of man and monkey compared with those in the other species.

The alkaline phosphatase activity of the glomeruli was particularly low in man and monkey; slightly higher levels were found in the dog and rabbit, whereas considerable activity was found in the rat glomeruli. Particularly high levels of activity were observed in the glomeruli of *Rana pipiens*, in which species there was higher activity in the glomeruli than in any other part of the nephron. This observation is similar to that reported by Pillai and Iyengar, who found, by histochemical staining techniques, that alkaline phosphatase activity was higher in the glomeruli than in the proximal tubules of *Rana hexadactylia* (12).

The alkaline phosphatase activity of proximal convolutions was significantly greater than that of distal convolutions in all species studied (Table II). Although this confirms in general the results obtained by histochemical staining techniques, the differences observed were less striking. For example, alkaline phosphatase activity is not usually detected in distal convolutions by histochemical staining techniques, whereas the ultra-microchemical technique revealed low but definite activity in this part of the nephron. Significant activity was

TABLE II

Alkaline phosphatase activity in the anatomical units of nephrons from healthy kidneys in man and in various animal species (expressed in moles of substrate split per kilogram dry weight of tissue per hour at 37° C) *

Structure analyzed	Man 6‡	Monkey 2‡	Dog 3‡	Rat 2‡	Rabbit 2‡	Frog 2‡	Toadfish† 5‡
Cortex:							
Glomeruli	0.56±0.072 [36]	0.60±0.081 [18]	1.90±0.15 [19]	9.70±0.88 [10]	1.03±0.25 [11]	29.8 ±3.80 [13]	Absent
Proximal convolutions	5.48±0.28 [40]	2.32±0.21 [24]	10.3 ±1.16 [27]	30.9 ±1.59 [21]	10.4 ±1.41 [13]	14.4 ±1.39 [16]	4.92±0.295 [37]
Distal convolutions	2.67±0.20 [41]	1.10±0.34 [3]	0.85±0.27 [17]	7.43±1.60 [9]	1.46±0.30 [9]	10.3 ±1.44 [12]	
Medullary ray							
(Superficial cortex)	2.55±0.15 [5]	4.88±0.43 [27]	0.66±0.13 [21]	39.7 ±2.88 [16]	5.85±0.81 [3]	Absent	Absent
(Deep cortex)	3.08±0.53 [5]	1.44±0.39 [11]			12.5 ±1.68 [10]		
Medulla:							
Outer medullary zone	0.81±0.13 [11]	0.36±0.13 [16]	2.25±0.65 [29]	50.1 ±4.50 [9]	13.8 ±1.32§ [9] 1.40±0.40‖ [12]	6.40±1.53 [10]	Absent
Inner medullary zone	1.27±0.11 [8]	0.25±0.11 [6]	0.70±0.18 [18]	3.65±0.68¶ [10] 3.20±0.25** [10]	0.10±0.037 [9]	8.70±1.73 [10]	Absent
Base of papilla	1.15±0.48 [6]	0.72±0.27 [5]	0.63±0.27 [3]	3.50±0.27 [9]	0.11±0.038 [10]	Absent	Absent
Apex of papilla	1.61±0.49 [9]	0.27±0.078 [11]	0.38±0.11 [6]	3.88±0.40 [10]	0.15±0.027 [5]	Absent	Absent
Artery	1.12±0.19 [25]	0.51±0.097 [19]	0.76±0.20 [12]	15.3 ±0.69 [13]	2.72±0.85 [5]	3.40±0.60 [16]	0.25±0.042 [7]

* Results are expressed as mean values with the standard error of the means. The total number of specimens of each structure analyzed is given in brackets.

† Toadfish, convoluted tubules only; lymphoid tissue = 30.9 ± 1.28, [19]; Wolff canal = 3.23 ± 0.15 MKH units, [27].

‡ Total number of kidneys analyzed.

§ Light tubules: descending limb of Henle's loop, collecting ducts and pars recta of proximal tubule.

‖ Dark tubules: ascending limb of Henle's loop.

¶ Dark tubules: Henle's loop.

** Light tubules: collecting ducts.

also found in the convoluted tubules of the aglomerular toadfish. Wilmer was unable to demonstrate any stainable alkaline phosphatase except in the interstitial lymphoid tissue of this species (13), but later work by Longley has demonstrated stainable alkaline phosphatase in the convoluted tubules of *Opsanus tau* (14).

Particularly high levels of activity were found in the glomerulus, in the proximal convolutions, the medullary ray, and outer medullary zone of the rat. The activity of the vessels in the rat was also high, and thus was not due to contamination from blood within the vessels.

In the monkey higher levels of alkaline phosphatase were found in the medullary ray near the capsule than in the deeper regions of the cortex. The reverse was true in the rabbit, but in other species no such differences were observed.

In the rat we were able to dissect the loop of Henle from the collecting tubules in the inner medullary zone (dark bar and light bar, respectively, in Figure 2). No significant difference was found in their alkaline phosphatase activity.

In the outer medullary zone of the rabbit kidney, the ascending loop of Henle (dark bar, Figure 2) was dissected from the descending loop of Henle and from the collecting tubules (light bar, Figure 2). In this species the mixture of collecting tu-

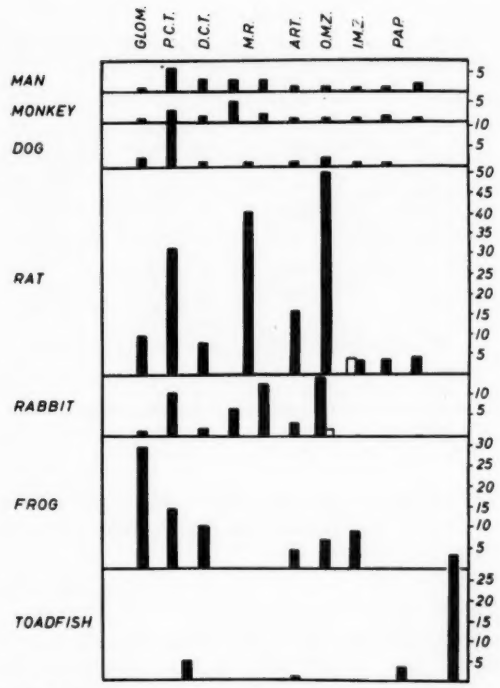

FIG. 2. THE QUANTITATIVE DISTRIBUTION OF ALKALINE PHOSPHATASE ACTIVITY IN THE NEPHRON. The bars represent mean alkaline phosphatase activities expressed in moles of substrate split per kilogram dry weight of tissue per hour at 37° C. Note that the same scale is used for all animal species studied. (See text and Table II for details.)

bules and descending limb of Henle's loop was found to have much greater alkaline phosphatase activity than the ascending limb of Henle's loop.

DISCUSSION

Techniques. When Lowry (4) developed his microtechniques for the study of brain cells, he first froze and then mounted the tissue specimens in two separate operations. In the case of the small fragments of tissue obtained by needle biopsy, it was not possible to mount the frozen specimen on a microtome sample holder without allowing the specimen to warm up appreciably. After trying several techniques unsuccessfully, we found that the two operations of freezing and mounting could be combined successfully into one. The tissue was placed directly onto a sample holder covered with a layer of 5 per cent tragacanth gel and the sample holder with the tissue already mounted was then immediately immersed in liquid nitrogen. Using this technique there was no visible evidence of ice crystal formation in biopsy material because the diameter of the sample was so small that rapid freezing could take place. When surgical biopsies and postmortem material were used, it was necessary to use slices of tissue less than 2 mm thick, to avoid harmful ice crystal formation.

Initially we employed sections cut at 30 μ, but these were too thick to allow satisfactory recognition of individual tubules. Sections cut at 6 to 10 μ were difficult to handle and provided too small a piece of tissue for analysis. These disadvantages did not occur with sections cut at 16 μ.

Most workers freeze-dry specimens at or below $-40°$ C (15, 16), because a uniform quality of preservation of tissue is thereby obtained. We found that kidney tissue could be frozen-dried at $-20°$ C without noticeable shrinking of the tissue. This had the advantage of allowing the freeze-drying process to be carried out in the cryostat in which the tissue was sectioned. Moreover, the drying process took place more rapidly at $-20°$ C, being complete in 4 to 5 hours.

Identification of individual parts of the nephron, particularly the proximal and distal convolutions, proved to be difficult in unstained frozen-dried tissue. McCann approached this problem by injecting trypan blue *in vivo,* and dissecting out separately the areas of proximal convolutions stained with trypan blue and unstained distal convolutions (8). As it was necessary to develop a technique applicable to human tissue, and as the dosage of trypan blue needed to outline the proximal convolutions would have resulted in a blue patient, McCann's technique was unsuitable for our needs. Moreover, renal biopsies in man could not necessarily be obtained at the time when there was optimal concentration of trypan blue in the proximal convolutions. For these reason we tried fluorescence, phase contrast, and polarization microscopy of the frozen-dried sections. With all these techniques no clear-cut differences were observed between proximal and distal convolutions at the low magnifications necessary for dissection. Thus far the only technique which allowed us to distinguish clearly between proximal and distal convolutions was the alternate section technique we used in this study. In addition, this technique allowed us to recognize and dissect separately

tubules in pathological human material, which showed various degrees of histological changes (17, 18).

It has been shown previously by conventional histochemical staining techniques in many species (19, 20) and with the trypan blue technique used by McCann in dogs (8) that the alkaline phosphatase activity of proximal convolutions was greater than that of distal convolutions. Our results confirm these findings since they demonstrate a consistently and significantly higher alkaline phosphatase activity in proximal than in distal convolutions. This suggests that our technique for identification and dissection of the individual parts of the nephron was accurate.

The validity and reproducibility of the quantitative histochemical technique used in these experiments has been demonstrated by Lowry and associates (6, 21). The sample size used in our studies was of the order of 15 to 100 mμg dry weight, which is slightly larger than that used by Lowry, Roberts and Chang (21) in their study of single brain cells (8 to 25 mμg). The average relative standard error of the mean for three replicate analyses of each of eight homogenates of human kidney was 1.49 per cent; for lactic dehydrogenase activity, the average relative standard error of the mean for four replicate analyses of each of eight homogenates was 2.31 per cent. These values are comparable to the average relative standard error of the means (10.5 per cent) for three different enzymes studied by Lowry and co-workers. In our experiments on dissected portions of the nephron the average relative standard error of the means for alkaline phosphatase was 15.4 per cent (Table III). For lactic dehydrogenase (22), the average relative standard error of the means was considerably smaller (8.3 per cent) (Table III). This difference between the standard errors of the means for alkaline phosphatase and lactic dehydrogenase can be accounted for partly by the fact that the readings for alkaline phosphatase were comparatively close to the blank values, whereas those for lactic dehydrogenase were well above the blank values. In this respect, it is significant that the average relative standard error of the means for alkaline phosphatase was only 8.8 per cent in the proximal convolutions, which had the highest activity. An alternate explanation is that the variation of enzyme activity from nephron to nephron is greater for alkaline phosphatase than for lactic dehydrogenase.

TABLE III

Average relative standard errors of the means for alkaline phosphatase and lactic dehydrogenase activities (all species combined) *

Structure analyzed	Alkaline phosphatase	Lactic dehydrogenase
	%	%
Glomerulus	13.5	6.9
Proximal convolutions	8.8	6.3
Distal convolutions	19.2	6.2
Medullary ray	14.4	12.3
Outer medullary zone	17.4	9.9
Inner medullary zone	13.8	8.5
Papilla	28.4	8.0
Over-all average	15.4	8.3

* Values for the arteries are not included in this table because the arterial tissue examined was not homogenous and consisted of arteries of different dimensions, varying from interlobular arteries to terminal branches.

Many authors, using histochemical staining techniques, have found no evidence of stainable alkaline phosphatase activity in the distal convolutions of many species (23). Our data indicate that there is a small but definite alkaline phosphatase activity in the distal convolution. The difference in enzyme activity demonstrated by these two techniques could be the result of inaccuracies in the dissection technique, resulting in intermixing of proximal and distal convolutions. However, this is unlikely for the following reasons: *1*) Significant alkaline phosphatase activity was found in the glomeruli, loops of Henle, collecting tubules and vessels—parts of the nephron in which no stainable alkaline phosphatase activity has been found (24, 25). In dissecting the glomeruli, papillary collecting tubules and vessels, there was virtually no opportunity for obtaining an admixture of structures with high activity. *2*) Our data are comparable to those obtained by McCann for the dog (8)—the only other quantitative study of alkaline phosphatase distribution in the nephron. McCann found that the ratio of activity in the distal to that of the proximal convolutions in the dog was 0.19. He considered that his samples of proximal convolutions contained about 10 per cent of distal convolutions and vice versa. When the ratio of activity is corrected on the basis of complete homogeneity of samples, a ratio of 0.081 is obtained, which is

virtually identical with the ratio of 0.083 found in our experiments.

The inherent limitations of the histochemical staining technique offer an alternative and more likely explanation for the discrepancies observed between the staining technique and the quantitative method. Visualization of stainable alkaline phosphatase depends on the product of activity and incubation time exceeding an indefinable threshold value for recognition by light microscopy. If the incubation time is so chosen as to obtain suitable staining of structures with high activity, such as the proximal convolutions, the threshold value may not be reached in structures with lower activity, such as the distal convolutions. The fact that measurable alkaline phosphatase activity was found in all parts of the nephron, including those in which stainable alkaline phosphatase has not been demonstrated, suggests that the differences between the two techniques are due to the inherent limitations of the histochemical staining technique. This view gains further support from a comparative study of the alkaline phosphatase distribution in the developing hamster tooth (26). When the alkaline phosphatase activity was measured quantitatively by the ultra-microtechniques used in this paper, the range of activity found in the various structures taken from the same dried section was from 0.85 ± 0.20 to 298 ± 26 moles of *p*-nitrophenylphosphate split per kg dry weight per hour (MKH units) at 37° C. If the adjacent section was stained for alkaline phosphatase by the Gomori technique under conditions optimal for structures having an activity of 298 MKH units, structures with an activity of 140 MKH units exhibited unequivocal staining for alkaline phosphatase, but an adjacent structure with an activity of 85 MKH units showed minimal stainable alkaline phosphatase even when the tissue was incubated for periods varying from 1 to 10 minutes. Structures with activities of 21 MKH units or less always gave a negative reaction under these conditions.

These experiments demonstrate that the quantitative measurement of enzyme activity provides accurate data on a wide range of activities in all structures of tissue, provided they can be dissected out accurately. By contrast, the standard histochemical staining techniques demonstrate qualitatively a comparatively narrow range of activities, and may give negative results for structures with appreciable levels of enzyme activity.

SUMMARY

1. A quantitative technique for enzyme assay of individual functional units of the nephron is described. This technique depends on: *a*) exact identification of individual parts of the nephron, using stained frozen sections; *b*) microdissection of the individual parts of the nephron in adjacent unstained frozen-dried sections; *c*) weighing and subsequent enzyme assay.

2. The optimal assay conditions for the alkaline phosphatase activity of the human kidney were determined. It was found that the enzyme was of the magnesium-activated, cyanide-inhibited type.

3. The morphology of frozen-dried unstained kidney sections of man, monkey, dog, rat, rabbit, frog and toadfish is described.

4. The quantitative distribution of alkaline phosphatase activity in the nephron of these species is reported. In general, alkaline phosphatase activity was found in all parts of the nephron analyzed, although there was considerable species variation. The highest activities were found consistently in the proximal convolutions, and the activities in the proximal convolutions were consistently significantly higher than those in the distal convolutions.

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QUANTITATIVE HISTOCHEMISTRY OF THE NEPHRON. III. LACTIC DEHYDROGENASE ACTIVITY IN MAN AND OTHER SPECIES *

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In previous papers (1, 2) we have described observations on the quantitative distribution of alkaline phosphatase in the anatomical units of the nephron in man and other species. This enzyme was selected for study by microdissection of lyophilized sections of kidneys and ultra-microassay because there was considerable information available on its qualitative distribution in the nephron. In particular, the known qualitative differences in its distribution in the proximal and distal convolutions of the tubule allowed us to verify the accuracy of the microdissection technique we used (2). Once this was accomplished we were able to study the quantitative distribution of other enzymes for which less information was available. In this paper the results of assay of lactic dehydrogenase (LDH) activity on renal tissues from healthy humans and laboratory animals are reported. LDH was chosen for study because it plays an important role in energy metabolism, namely, in the glycolytic formation of lactic acid and in its mobilization for complete oxidation in the tricarboxylic acid cycle. As far as could be ascertained, no data were available on its distribution or functional significance in the cells of the nephron.

METHODS

Handling of tissue. In previous communications (1, 2) we have described in detail the techniques used for dissecting, weighing, and enzyme assay of the individual units of the nephron.

LDH assay. Optimal conditions for assay of LDH activity in the human kidney were determined (Table I). Tissue samples with a dry weight ranging from 10 to 100 mμg were inserted into 10 μl of a substrate medium containing 1.0 mM sodium pyruvate, 1.25 mM reduced diphosphopyridine nucleotide (DPNH, Sigma), 25 mM nicotinamide, and 0.05 per cent bovine serum albumin (Armour) in 0.2 M Tris buffer, pH 7.15. After incubation for 30 minutes at 37° C the reaction was stopped and the excess DPNH destroyed by adding 2 μl 1.0 N HCl. A 10 μl aliquot was then transferred to a 75 × 10 mm Pyrex tube containing 100 μl 6.5 N NaOH and incubated for 30 minutes at 37° C. The oxidized DPN formed by enzyme action during the first incubation period was thereby converted into a fluorescent substance (3). After dilution of the resulting solution with 1.0 ml H_2O the fluorescence was determined in a Farrand filter fluorimeter (primary filter, Corning glass color filter no. 5860; secondary filters, Corning nos. 4308, 5562, 3387). All readings were made against a quinine standard (0.25 μg per ml). The results were expressed in moles of DPNH oxidized per hour at 37° C per kilogram dry weight of tissue (MKH units).

Effects of dry and wet "canning" on LDH activity. Because Lowry, Roberts and Chang (4) have shown that the method of "canning" (i.e., the act of inserting lyophilized cells or tissue fragments into the microtest tubes prior to incubation) may affect enzyme assays, we studied the effects of two methods of canning on the assay of LDH. In dry canning the weighed tissue fragments were inserted into clean dry microtest tubes. The rack

TABLE I

Properties of lactic dehydrogenase in the human kidney

Michaelis-Menten constant	Pyruvate DPNH	0.20 mM 0.11 mM
Optimal substrate concentration	Pyruvate DPNH	1.0 mM 2.5 mM
Optimal pH	7.15	
Activity vs incubation time (37° C)	Linear 0 to 120 minutes	
Activity vs enzyme concentration	Linear between 0.0 and 0.35 mmole/hour/L of incubation mixture	
$V_{2°\,C}/V_{37°\,C}$*	0.036	

* V = velocity of the enzyme reaction at the temperatures given.

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† Established Investigator, American Heart Association, supported by the Illinois Heart Association.

TABLE II

The effect of the method of "canning" on the lactic dehydrogenase activity *

Structure analyzed	Monkey			Dog		
	Wet	Dry	$\frac{\text{Dry}}{\text{Wet}} \times 100$	Wet	Dry	$\frac{\text{Dry}}{\text{Wet}} \times 100$
Glomerulus	55± 5.6 [13]	44± 5.8 [13]	80	82± 9 [6]	70± 4 [5]	85
Proximal convoluted tubules	78± 4.2 [13]	78± 4.9 [14]	100	353± 2.7 [5]	151±20 [8]	43†
Distal convoluted tubules	116±10.6 [13]	90± 8.2 [10]	78	366±37 [6]	252±31 [4]	68†
Medullary ray	137± 6.6 [26]	143± 6.6 [18]	104	316±14 [12]	127± 6.1 [6]	40†
Outer medullary zone	171±11.0 [10]	167±20.4 [8]	98	280±11 [6]	63± 6.7 [8]	22†
Inner medullary zone	146± 5.5 [18]	135± 5.0 [4]	93	125± 6.5 [6]	43± 2.5 [6]	34†
Papilla	63± 4.6 [10]	27± 6.5 [4]	43†	39± 3.5 [10]	16± 4.5 [10]	41†
Vessel	51± 5.4 [13]	44± 4.4 [12]	86	22± 0.6 [3]	10± 2.0 [6]	45†

* Expressed in moles DPNH oxidized per hour at 37° C per kilogram dry weight. The total number of specimens of each structure analyzed is given in brackets.

† Significantly below 100 at 0.05 level.

with tubes was placed in an ice bath and the incubation medium was then added. In wet canning the weighed tissue specimens were inserted directly into the substrate medium previously pipetted into microtest tubes, which were kept in an ice bath until all specimens had been inserted.

Intracellular distribution of LDH activity. The intracellular distribution of LDH activity was also studied because we were unable to find such data in the literature. A 10 per cent homogenate was made of whole rat kidney in 0.25 M sucrose containing 1.8 mM $CaCl_2$ and subjected to fractionation in the cold using a Spinco preparative ultracentrifuge (5). The resulting fractions were assayed for LDH activity. The nuclear fraction was prepared by layering 6 ml homogenate on 4 ml 0.34 M sucrose containing 0.18 mM $CaCl_2$ and centrifuging at $100 \times G$ for 10 minutes. The mitochondrial fraction was isolated by centrifuging the supernatant at $5{,}000 \times G$ for 10 minutes; the sediment was washed, resuspended, and centrifuged for 10 minutes at $24{,}000 \times G$. The microsomal fraction was prepared from the supernatant remaining after sedimentation of the mitochondrial fraction by centrifugation for 1 hour at $50{,}000 \times G$. The supernatant remaining after this centrifugation was the supernatant fraction.

RESULTS

Technical

Table II shows the effects of two methods of "canning" on LDH activity. From this it is clear that the wet canning method gave significantly higher results for LDH activity than did the dry canning method in half of the cases. The wet canning technique was therefore adopted as a routine, and the incubation was carried out directly after the canning was completed.

Experimental

Intracellular distribution. Data on LDH assay in the fractions of renal homogenates are given in Table III. It can be seen that 93.3 per cent of the LDH activity was found in the supernatant fraction, while the total for the activities of the individ-

TABLE III

The intracellular distribution of lactic dehydrogenase in whole rat kidney homogenates *

Fraction analyzed	Percentage of activity in homogenate†
Homogenate	100 ± 0.26
Nuclear fraction	1.15 ± 0.40
Mitochondrial fraction	6.60 ± 1.90
Microsomal fraction	3.35 ± 0.71
Supernatant fraction	93.3 ± 1.8
Total	104.4 ± 2.7

* These are combined results from 3 separate fractionations on 6 rat kidneys, each fraction being analyzed in triplicate.

† Results are mean values with standard errors of the mean.

TABLE IV

Lactic dehydrogenase activity in the anatomical units of nephrons from healthy kidneys in man and various animal species (expressed in moles of DPNH oxidized per kilogram dry weight of tissue per hour at 37° C) *

Structure analyzed	Man 7‡	Monkey 2‡	Dog 3‡	Rat 2‡	Rabbit 2‡	Frog 2‡	Toadfish† 5‡
Cortex:							
Glomeruli	106± 6.1 [46]	55.0± 2.66 [13]	73.6± 3.78 [17]	71.3± 5.55 [12]	55.2± 5.14 [17]	42.7±3.76 [14]	Absent
Proximal convolutions	261± 9.3 [55]	76.6± 1.97 [13]	256 ±15.8 [19]	152 ± 8.06 [14]	83.3± 8.24 [13]	62.5±4.44 [13]	28.5±4.24 [38]
Distal convolutions	261± 9.9 [49]	114 ± 5.77 [13]	356 +32.1 [18]	231 ±18.3 [17]	289 ±18.3 [13]	83.6±3.12 [15]	
Medullary ray							
(Superficial cortex)	227± 9.6 [25]	129 ± 3.77 [13]	280 ±17.5 [31]	104 ±27.4§ [11]	170 ±54.1 [12]	Absent	Absent
(Deep cortex)		148 ± 4.19 [13]		159 ±15.5‖ [4]			
Medulla:							
Outer medullary zone	200±12.0 [11]	170 ± 5.41 [10]	182 ±20.5 [20]	311 ±58.4¶ [3] 88.4±25.7§ [9]	129 ±12.6** [8] 309 ±19.8¶ [12]	72.2±7.38 [13]	Absent
Inner medullary zone	130± 5.51 [11]	156 ±16.0 [4]	155 ±10.3 [15]	219 ±38.9¶ [10] 66.8± 4.60†† [9]	295 ±10.8 [12]	79.4±7.70 [12]	Absent
Base of papilla	158±24.3 [8]	65.4± 5.50 [4]	87.4±10.9 [10]	182 ± 5.01 [2]	181 ± 8.5 [5]	Absent	Absent
Apex of papilla				293 ±11.0 [6]			
Artery	131±12.8 [31]	49.2± 2.89 [13]	61.5±13.1 [10]	66.0± 5.10 [9]	47.0± 8.3 [7]	27.1±6.30 [13]	39.5±3.38 [19]

* Results are expressed as mean values with the standard error of the means. The total number of specimens of each structure analyzed is given in brackets.

† Toadfish, convoluted tubules only; lymphoid tissue = 83.4 ± 2.99 [42]; Wolff canal = 67.3 ± 4.07 MKH units [37].

‡ Total number of kidneys analyzed.

§ Light: pars recta and collecting tubules.

‖ Dark: distal convolutions.

¶ Dark: ascending loop of Henle.

** Light: descending loop of Henle and collecting tubules.

†† Light: collecting tubules.

ual fractions was very close to the activity of the whole homogenate.

Individual nephrons. LDH activity was assayed in the individual units of the nephron of man, monkey (*Macacca mulatta*), dog (mongrel), rat (Sprague-Dawley), rabbit (New Zealand white), frog (*Rana pipiens*), and aglomerular toadfish (*Opsanus tau*). The results are shown in Table IV. The number ot individuals or animals studied and the number of fragments analyzed are given, together with the mean values and standard error of the means. The quantitative distributions are shown diagrammatically in Figure 1.

The over-all level of LDH activity was high, being approximately from 10 to more than 100 times the activity obtained for alkaline phosphatase (*2*).

In man no difference was found between the LDH activities in the proximal and distal convoluted tubules. In all other species the activity in the distal convoluted tubules was greater than that in the proximal convoluted tubules. The levels of activity found in the medullary tubules were comparable to those in the cortical tubules. Except for the vessels, the lowest LDH activity was found in the glomeruli of all species.

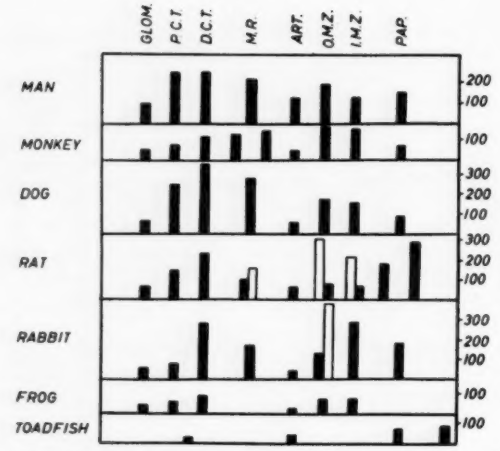

FIG. 1. THE QUANTITATIVE DISTRIBUTION OF LACTIC DEHYDROGENASE ACTIVITY (LDH) IN THE NEPHRON. The bars represent mean LDH activities expressed in moles of DPNH split per kilogram dry weight of tissue per hour at 37° C. (See text and Table IV for details.)

After the experiments reported here were completed, further assays of LDH activities were made in our laboratory. All the results obtained were from 29 to 42 per cent of those reported in Table IV; however, the distribution and ratio of LDH activity in the individual units of the nephron remained the same. The over-all decrease in LDH activity was found to coincide with the use of a new batch of DPNH (Sigma). An investigation planned to find the cause of the discrepancy was seriously handicapped by the fact that none of the first batch of DPNH was available for study. However, a thorough study was made of all possible causes of inhibition of LDH activity which suggested themselves to us.

Whereas with the first batch of DPNH the LDH activity in the rat glomerulus was 71.3 ± 5.55 MKH units, the activity found in the new batch of DPNH was 29.3 ± 2.71 MKH units, i.e., a ratio of 2.42 to 1. Similarly, the LDH activity of normal human glomeruli was found to be 106 and 30.8 MKH units with the first and second batches of DPNH, respectively, i.e., a ratio of 3.45 to 1. Recently Dubach and Recant have published a value of 26.6 ± 3.0 MKH units for the rat glomerulus (6), which agrees closely with values for LDH activity we are getting at present. This might suggest either that an inhibitor of renal LDH activity was present in the second batch of DPNH, or that an activator was present in the first batch.

DISCUSSION

Technique. Since higher results were obtained with the wet canning technique than with the dry canning technique, wet canning was employed in all the LDH assays. Lowry and associates have shown that the loss of activity in the dry canning technique was due to condensation of water on the small fragment of tissue before the incubation medium was added (4). Two sets of experiments were performed to compare the effects of the two canning methods. The experiment on monkey kidney was done while the relative humidity was 10 per cent. The loss of activity in the dry canning method did not exceed 22 per cent, except in the case of the papilla, which was in the ice bath for the longest period before the reagent was added. The experiment was done on dog kidney while the relative humidity was 25 per cent; the loss of LDH activity in the dry canning method was significant in all structures except the glomeruli, and exceeded 50 per cent in all but two structures. These data confirm Lowry and co-workers' suggestion (4) that condensation of water on the minute specimen is the important factor in the loss of enzyme activity in the dry canning method. It also seemed possible that the humidity of the atmosphere might affect the enzyme activity during the 2 to 3 hours' exposure of the tissue prior to canning, i.e., during dissection and weighing. For this reason we compared the activities found in proximal and distal convoluted tubules for normal man with the relative humidity prevailing when the experiments were done. Over a range of relative humidity from 22 to 40 per cent no correlation existed between the humidities prevailing and the LDH activities.

One further factor which might possibly influence the results is the length of storage of the tissue sections prior to assay. We found that storage up to six months of the frozen-dried tissue sections *in vacuo* at − 35° C did not influence the enzyme activity. This is in accord with the results reported by Strominger and Lowry (7).

The validity and reproducibility of the quantitative histochemical technique used in these experiments (1) have been demonstrated by Lowry and associates (8). The sample size used in our stud-

ies was of the order of 15 to 100 mμg, which is slightly larger than that used by Lowry and coworkers in their study of single brain cells (8 to 25 mμg) (4). The average relative standard error of the mean in all our experiments was 8.3 per cent. This value is comparable to the average relative standard error of the mean (10.5 per cent) for three different enzymes studied by Lowry and colleagues.

Experimental. The results for LDH activity were much higher on a molar basis than those found for alkaline phosphatase in the same species (2), and also higher than those found for aldolase, fumarase, hexokinase, and phosphohexoisomerase in the dog kidney (9). Thus, assuming these measurements quantitatively reflect activity *in vivo*, it seems unlikely that LDH is a rate-limiting enzyme in the glycolytic system in any part of the nephron.

LDH was more evenly distributed throughout the units of the nephron than was alkaline phosphatase. The lowest levels of LDH activity were found in the glomeruli. This appears to be part of a general pattern emerging from the data reported by McCann for aldolase, fumarase, hexokinase and phosphohexoisomerase in the dog kidney (9), and from our own unpublished results with glucose-6-phosphatase, adenosine triphosphatase and hexokinase (10).

The finding that LDH is almost exclusively located in the nonparticulate cytoplasm is noteworthy in view of the fact that the enzymes of the Krebs cycle have been located principally in the mitochondria (11, 12), whereas the nonparticulate cytoplasm contains the glycolytic system (11). This would mean that it is necessary for lactic acid to be oxidized to pyruvic acid in the nonparticulate cytoplasm before entering the Krebs cycle in the mitochondria.

SUMMARY

1. The quantitative distribution of lactic dehydrogenase (LDH) activity in the nephron of man, monkey, dog, rat, rabbit, frog and toadfish was studied.

2. Considerable LDH activity was found in all parts of the nephron analyzed. In all cases the cortical tubules had higher activity than did the glomeruli. The highest levels of activity were found in the nephrons of man and dog, the lowest in the frog and the toadfish.

3. Within the cell, LDH activity was found almost exclusively in the nonparticulate cytoplasm.

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QUANTITATIVE HISTOCHEMISTRY OF THE NEPHRON. IV. ALKALINE PHOSPHATASE AND LACTIC DEHYDROGENASE ACTIVITIES IN RENAL TUBULAR DISEASES *

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The present investigations were designed to study by a quantitative technique the distribution of enzyme activity in the various individual anatomical and functional units of the human nephron. Our first objective was to study the role played by enzymes in the formation of urine, particularly in the secretion and reabsorption of various substances in the renal tubule. The second objective was to discover enzymes in the kidney which may appear in the blood stream or in urine, and whose measurement in renal disease could be used as an index of damage to specific structures in the nephron.

In this paper investigations are reported on the relationship between isolated and combined defects in the function of the renal tubules and alkaline phosphatase and lactic dehydrogenase (LDH) activity in the nephron. Assays of enzyme activity were made on the individual anatomical units of the nephron of kidneys from patients with familial renal glycosuria, hypophosphatasia, phosphate-losing renal tubular disease, cystine storage disease, and the adult Fanconi syndrome. The results were compared with those from kidneys of persons free of renal disease.

MATERIAL AND METHODS

Healthy kidneys. In five instances specimens of healthy kidneys were taken by biopsy during the course of a laparotomy for disease unrelated to the kidney, e.g., during elective cholecystectomy or repair of a ventral hernia. Two specimens were taken at autopsy, 3 and 6 hours after death. All of the kidney specimens so obtained were from patients with no recent or past history of renal disease, and in whom examination of the urine and blood revealed no evidence of renal disease. In none was the primary disease one known to have secondary effects on the kidney.

Abnormal kidneys. The renal tissue was removed by percutaneous renal biopsy (1) from 5 patients (see below). Tissue was also obtained postmortem from a patient with the adult Fanconi syndrome (Case 1) and from an infant with the infantile form of hypophosphatasia. These specimens were obtained and frozen within 1 and 5 hours after death, respectively. Two specimens of kidney tissue were analyzed from the patient with the phosphate-losing renal tubular disease, who died suddenly from a subarachnoid hemorrhage. A specimen had been obtained by biopsy 11 weeks before death, the second was taken at autopsy 4 hours after she died.

Methods of dissection and analysis of tissue for enzyme activity. These have been described in detail in previous communications from this laboratory (2, 3). Optimal conditions for the assay of alkaline phosphatase and LDH activities in human kidneys were used for the assays (2, 3). The results were expressed in moles of substrate split or in moles of DPNH oxidized per kilogram dry weight of tissue per hour at 37° C.

RESULTS

Statistical handling of data (Tables I and II)

In the previous two papers the results of enzyme assays were expressed in terms of the total number of fragments of each structure studied (2, 3). A different form of analysis was necessary in the present study in order to compare data from single pathological cases with data from analyses of several normal kidneys. Therefore, in the case of healthy kidneys the results were expressed as means with standard deviations of the means of all the healthy kidneys grouped together. The results for the single pathological specimens were expressed differently—as means with standard deviations of the *individual* renal fragments analyzed.

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† Established Investigator, American Heart Association, supported by the Illinois Heart Association.

TABLE I

Alkaline phosphatase activity in the anatomical units of nephrons from healthy kidneys and from the kidneys of patients with renal tubular dysfunction (expressed in moles of substrate split per kilogram dry weight of tissue per hour at 37° C) *

Structure analyzed	Healthy adult kidneys *6*†	Familial renal glycosuria	Hypophosphatasia: Juvenile Case 1	Hypophosphatasia: Juvenile Case 2	Hypophosphatasia: Infantile	Phosphate-losing renal tubular disease‡	Cystine storage disease	Adult Fanconi syndrome Case 1	Adult Fanconi syndrome Case 2	Adult Fanconi syndrome Case 3
Glomeruli	0.48±0.17 *5* [36]	0.70±0.07 [5]	0.16±0.20 [5]	0.32±0.28 [6]	0.10±0.098 [6]	0.32±0.21 [14]	0.90±0.58 [7]	0.40±0.40 [7]		0.21 ±0.11 [7]
Proximal convolutions	5.70±0.90 *5* [40]	2.60±0.81 [9]	0.54±0.40 [7]	0.49±0.12 [7]	0.20±0.20§ [8]	1.40±0.42 [18]	7.20±1.99 [5]	0.35±0.21 [7]	2.00±0.60 [9]	0.95 ±0.11 [8]
Distal convolutions	2.70±0.50 *6* [40]	1.20±0.61 [7]	0.30±0.25 [5]	0.37±0.21 [6]		1.21±0.93 [12]		1.10±0.50 [4]	0.13±0.23 [2]	0.35 ±0.12 [7]
Degenerated convolutions						0.92±0.81 [11]	1.20±0.58 [7]		0.40±0.29 [6]	
Medullary rays	2.84 *1* [9]	2.70±1.42 [8]	0.79±0.21 [5]	0.34±0.20 [6]	0.30±0.29 [6]	1.10±0.45 [24]	2.00±0.40‖ [3] 0.80±0.40¶ [3]		0.60±0.59 [6]	2.06 ±0.18 [7]
Medulla (outer and inner medullary zones)	0.80±0.20 *2* [11]				0.50±0.81 [5]	0.70±0.29 [12]		0.60±0.79 [13]		0.60 ±0.41 [14]
Papilla	1.70±0.89 *2* [15]					0.37±0.13 [6]		0.50±0.29 [5]		
Small artery	1.20±0.70 *5* [25]		0.17±0.19 [3]	0.15±0.054 [3]	0.10±0.089 [5]	0.14±0.086 [7]	0.60±0.19 [7]	0.50±0.29 [6]	0.40±0.40 [7]	0.092±0.086 [11]

* For the healthy kidneys the mean value and standard deviation are given for the averages of each patient; for the pathologic kidneys the mean value and standard deviation are given for the fragments of tissue analyzed. The total number of tissue fragments analyzed is given in brackets.

† Number of kidneys analyzed in each group is shown in italic type.

‡ Pooled data from one biopsy and one autopsy specimen from the same patient; no significant differences were found between these specimens.

§ Proximal and distal convolutions could not be dissected out separately in this patient.

‖ Histologically normal medullary ray.

¶ Histologically atrophic and degenerated medullary ray.

Values removed more than 2 standard deviations from the mean values obtained for healthy kidneys were regarded as significantly different statistically ($p = < 0.05$). In evaluating the functional significance of changes in enzyme activity, however, it must be kept in mind that very considerable changes in enzyme activity may be necessary before functional effects can be expected.

Healthy kidneys

Alkaline phosphatase. The level of alkaline phosphatase activity was found, on a molar basis, to be low throughout the nephron, the lowest activity being in the glomeruli (Table I). The highest activity was found in the proximal convolutions, and this activity was significantly greater than that in the distal convolutions ($t = 6.65$; $p = < 0.001$). Comparatively low levels of activity were found in the medulla and papilla.

LDH. There was a high level of activity, on a molar basis, of this enzyme throughout the nephron (Table II). The enzymatic activity of the proximal and distal convolutions was higher than that of any other segment of the nephron, and as in the case of alkaline phosphatase, the lowest activity was found in the glomeruli. In the case of both enzymes there were no significant differences in activity between specimens taken by biopsy and at autopsy.

Results in disease states

Renal glycosuria. A 31 year old bank clerk and many members of his family were known to have renal glycosuria. He was in excellent health and fasting blood glucose levels and oral glucose tolerance tests were normal. The renal functions measured were within normal limits, as was the histology of the renal biopsy.

Alkaline phosphatase activity was decreased in the proximal (> 3 SD) and in the distal convolutions ($= 3$ SD), but was not significantly altered in the glomeruli. The LDH activity was not significantly different from normal in any unit of the nephron analyzed.

Hypophosphatasia. Although hypophosphatasia is not usually considered to be a disease with clinically significant renal tubular dysfunction, it was chosen for study because of the possible relationship between alkaline phosphatase activity and the reabsorption of glucose by the proximal tubule (4, 5). Kidneys from three children with hypophosphatasia were studied. Full details of the three cases have been published elsewhere (6).

The first patient, a two and one-half year old

TABLE II

Lactic dehydrogenase activity in the anatomical units of nephrons from healthy kidneys and from the kidneys of patients with renal tubular dysfunction (expressed in moles of DPNH oxidized per kilogram dry weight of tissue per hour at 37° C) *

Structure analyzed	Healthy adult kidneys 7†	Familial renal glycosuria	Hypophosphatasia			Phosphate-losing renal tubular disease‡§	Cystine storage disease	Adult Fanconi syndrome		
			Juvenile		Infantile					
			Case 1‡	Case 2‡				Case 1	Case 2	Case 3‡
Glomeruli	108 ± 22 *6* [46]	116 ± 21.5 [8]	122 ± 32.4 [5]	142 ± 49.9 [6]	213 ± 44.1 [6]	146 ± 15.2 [6]	39 ± 7.06 [8]	58 ± 23.0 [7]	50 ± 0.99 [2]	87 ± 8.63 [7]
Proximal convolutions	257 ± 41 *7* [55]	299 ± 44.1 [6]	206 ± 10.2 [6]	312 ± 64.0 [7]		314 ± 15.0 [7]	171 ± 21.1 [21]	114 ± 26.8 [7]	165 ± 41.0 [8]	314 ± 51.0 [7]
Distal convolutions	256 ± 44 *7* [49]	334 ± 35.8 [5]	236 ± 52.6 [8]	299 ± 77.0 [6]	357 ± 33.1‖	336 ± 53.8 [7]		131 ± 63.0 [7]	191 ± 28.0 [7]	336 ± 53.8 [7]
Medullary rays	231 ± 25 *3* [25]	249 ± 34.4 [7]	234 ± 64.9 [6]	260 ± 45.6 [6]	375 ± 27.0 [6]	271 ± 69.0 [6]	134 ± 29.1 [7]	99 ± 23.0 [7]	137 ± 31.1 [6]	320 ± 59.4 [7]
Medulla (outer and inner medullary zones)	165 ± 21 *3* [22]				326 ± 30.8 [5]	289 ± 40.9 [12]		108 ± 24.0 [6]	152 ± 100.0 [10]	200 ± 26.9 [17]
Papilla	175 ± 82 *3* [8]					152 ± 30.3 [12]		89 ± 18.1 [6]		
Small artery	131 ± 61 *6* [31]		151 ± 24.8 [4]	196 ± 14.7 [5]	203 ± 39.2 [6]	77 ± 16.0 [6]	30 ± 13.1 [2]	53 ± 23.0 [6]	26 ± 7.08 [8]	62 ± 19.5 [6]

* For the healthy kidneys the mean value and standard deviation are given for the averages of each patient; for the pathologic kidneys the mean value and standard deviation are given for the fragments of tissue analyzed. The total number of tissue fragments analyzed is given in brackets.

† Number of kidneys analyzed in each group is shown in italic type.

‡ Multiplied with factor 3.45 to correct for inhibitor present in DPNH used in these experiments (3).

§ Autopsy specimen only.

‖ Proximal and distal convolutions could not be dissected out separately in this patient.

white girl, presented because of loss of the deciduous teeth. There were no abnormal clinical findings but changes consistent with those seen in hypophosphatasia were found on X-ray of the long bones. The serum alkaline phosphatase level was 0.5 Bodansky unit.

The second patient, a three year old white girl, had widespread bony deformities, premature loss of deciduous teeth, and radiological changes consistent with hypophosphatasia. The serum alkaline phosphatase level was 0.6 Bodansky unit.

The third patient, a two month old white infant, had had gross skeletal defects from birth. Seizures characteristic of hypsarrhythmia appeared at eight days. The child died of pneumonia. Physical abnormalities included gross skeletal deformities, with wide flat fontanelles and widely separated sutures. Radiologically, there was delayed ossification of the skull, demineralization of the spine, marked flaring of costochondral junctions and an irregular appearance of the rib shaft. The metaphyses were demineralized and there were deformities of the diaphyses of the long bones. The serum alkaline phosphatase was 0.0 and 0.7 Bodansky unit; calcium was 11 mg per 100 ml and inorganic phosphate was 5.2 mg per 100 ml. Overgrowth of cartilage, failure of bone resorption, much osteoid, poor calcification and irregularity of bone trabeculae were noted postmortem. Calcium deposits were found in the interstitial tissue of the kidney among normal glomeruli and tubules. The tissue alkaline phosphatase activities were measured in homogenates of liver, spleen, kidney and bone, and were found to be from 0.7 to 15 per cent of levels in tissues from an infant of the same age who had died in an accident (6).

Serum alkaline phosphatase levels were abnormally low in members of the families of all three children. Phospho-ethanolamine was demonstrated by two-dimensional paper chromatography in the urine of all three patients. None of the three patients had glycosuria. The first patient, however, was found to have aminoaciduria.

Extremely low levels of alkaline phosphatase activity were found in nephrons from these three patients, but the levels of activity in the two juvenile cases of hypophosphatasia were approximately double that found in the infantile. All these results were significantly lower than measurements from two normal infant kidneys, analyzed as controls (7). The LDH activity in the juvenile cases did not differ significantly from that found in the healthy adult or infant kidneys, but there was a slight increase of LDH activity in the case of infantile hypophosphatasia.

Phosphate-losing renal tubular disease. The kidney from a 53 year old white advertising executive was studied on two occasions. She had enjoyed excellent health until the age of 50, when she had a sudden pain in the chest and was found to have several fractured ribs. Thenceforth she sustained numerous spontaneous fractures. The serum calcium was between 9.5 and 10.8 mg per 100 ml, inorganic phosphate between 1.8 and 2.5 mg per 100 ml, and alkaline phosphatase from 3 to 4 Bodansky units. The tubular reabsorption of phosphate was 47 per cent on a diet containing 1.5 g phosphate per day. It was not affected by treatment with 500,000 units of vitamin D daily for 6 days, but it increased from 47 to 59 per cent when calcium (1.5 mg per kg) was infused. The urine specific gravity varied from 1.002 to 1.018, and the serum nonprotein nitrogen was 12 mg per 100 ml. There was no aminoaciduria or glycosuria. There was no evidence of intestinal malabsorption. Several bone biopsies were characteristic of osteomalacia, and the parathyroid glands were found to be normal on exploration of the neck and mediastinum (8). The patient died from a subarachnoid hemorrhage. No morphologic abnormalities were found in the renal biopsy specimen or in the kidneys examined postmortem.

The alkaline phosphatase activity was low throughout the nephron, but least abnormal in the glomeruli. These differences were significant in the proximal convolutions (> 4 SD) and in the distal convolutions ($= 3$ SD). Even lower activities were found in histologically degenerated convoluted tubules. The LDH activity was slightly but not significantly increased throughout the nephron, except in the papilla.

Cystine storage disease with Fanconi syndrome. Nephrons from one patient, aged four and one-half years, with cystine storage disease, were studied. Failure to thrive had been noted from the age of 18 months, and polyuria and polydipsia from the age of three years. Cystine crystals were found in the corneas, and in the reticuloendothelial cells of the bone marrow. The urine volumes varied between 2 and 5 L daily, and the urine specific

gravity did not exceed 1.005 during dehydration or after injection of Pitressin (vasopressin). There was no glycosuria but there was a pronounced aminoaciduria (total α-amino-nitrogen excretion 1,545 mg per 24 hours). The serum potassium was consistently low. On histological examination of the renal biopsy, slight degenerative changes were found in the convoluted tubules, and there was a slight increase of fuchsin-positive and periodic acid-Schiff (PAS)-positive granules in the cytoplasm. No other abnormality was found. The father, paternal grandfather and one sister were found to have a heavy aminoaciduria. The second sister was shown to have cystine storage disease; cystine crystals were seen in corneas and bone marrow, and rickets, aminoaciduria and glycosuria were noted.

The alkaline phosphatase activity was significantly higher than normal in the glomeruli (> 2 SD) and was slightly elevated in the histologically normal proximal convolutions (< 2 SD). However, the alkaline phosphatase activity was significantly decreased in the histologically degenerated convolutions. By contrast, LDH activity was significantly decreased in glomeruli (> 3 SD), proximal convolutions (= 2 SD) and medullary rays (> 3 SD).

Adult Fanconi syndrome. The kidneys from three patients with the adult Fanconi syndrome were studied. Full details of the clinical histories and metabolic studies on Patients 1 and 2 will be published elsewhere (9). Although there were considerable pathophysiological similarities in these three patients, we report them below individually because of the different underlying renal pathologies.

Patient 1, a 51 year old man, died in cardiac and hepatic failure with postnecrotic cirrhosis, osteomalacia and atypical multiple myeloma. During life the urine volume varied from 2 to 5 L, and the urine specific gravity from 1.012 to 1.018. The creatinine clearance was 55 ml per minute, the urea clearance was 60 per cent of average normal renal function, and the excretion of phenolsulfonphthalein was 15 per cent in 2 hours. The serum nonprotein nitrogen was 27 mg per 100 ml. The following substances were excreted in excess in the urine: glucose, glycine, α-amino-nitrogen, inorganic phosphate, calcium, uric acid, and potassium. Tubular reabsorption of phosphate was 21 to 38 per cent. On microscopic examination of the kidney, moderately severe degenerative changes were seen in the tubular cells, and there was patchy tubular atrophy. Several tubules contained hyaline or pigmented casts, and some of the casts contained bilirubin. In some tubules there was syncytial formation such as occurs in multiple myeloma. Cuboidal crystals of unknown composition were seen in proximal convoluted tubules and interstitial tissue. The glomeruli were normal but there was diffuse fibrosis of the interstitial tissue.

Patient 2, a 52 year old housewife, was ill with osteomalacia and multiple pseudofractures. Her urine volume varied from 1.5 to 2 L per day, the specific gravity from 1.012 to 1.017, and the pH from 6.0 to 7.5. The endogenous creatinine clearance was 21.4 ml per minute, the urea clearance was 22 per cent of average normal renal function, and the excretion of phenolsulfonphthalein was 12 per cent in 2 hours. The serum nonprotein nitrogen was 50 mg per 100 ml. She excreted the following substances in excess in her urine: glucose, glycine, α-amino-nitrogen, uric acid, sodium, chloride, potassium, and phosphate. Pyelonephritis and arterial and arteriolar nephrosclerosis were diagnosed by means of a renal biopsy. Several glomeruli were hyalinized and there was a moderate degree of tubular atrophy and degeneration.

Patient 3, a 38 year old Negro housewife, was also ill with osteomalacia and multiple pseudofractures, and in addition had metastases from an osteogenic sarcoma. The serum calcium ranged from 9.4 to 10.3 mg per 100 ml, and the inorganic phosphate from 1.1 to 1.8 mg per 100 ml. The serum alkaline phosphate level was normal. The urine volume varied from 1.4 to 2.0 L daily, and the specific gravity from 1.022 to 1.031. The endogenous creatinine clearance was 119 ml per minute, and the serum urea nitrogen was 8 mg per 100 ml. She excreted the following substances in excess in her urine: glucose, phosphate, glycine, and other amino acids. No abnormality was found on histological examination of the renal biopsy specimen.

Biochemical analyses of the kidneys from these three patients revealed a marked decrease of alkaline phosphatase activity throughout the nephrons, except for the glomeruli, where it was normal. Particularly low levels were found in the proximal and distal convolutions (> 3 SD). LDH activity

was also significantly decreased in the glomeruli, proximal convolutions and medullary rays of Patients 1 and 2, and in the distal convolutions of Patient 1. However, there was a slight increase of LDH activity throughout the nephrons of the third patient, but this was significant only in the medullary ray (> 3 SD).

DISCUSSION

Very little is known currently of the distribution and functions of enzymes in the individual anatomical and functional units of the nephron. The limited knowledge we possess of the distribution of enzyme activity in the nephron has been gleaned by histological staining techniques. Because these techniques yield only qualitative information of limited value, the quantitative techniques reported in the previous two papers (2, 3) were applied to the study of human renal tissue.

Method of dissection of specimens. The justification for and validity of the microdissection technique were discussed in a previous paper (2), in which it was shown that individual units of the healthy nephron could be dissected out accurately and assayed with precision for enzyme activity. Several new problems arose when nephrons from diseased kidneys were studied. In particular, there were considerable variations in the histological appearance both of glomeruli and of the individual anatomical units of the tubules. For example, the appearance of the proximal convolutions was more heterogeneous than that in healthy kidneys. Some appeared normal with brush borders, whereas others showed either degenerative changes or atrophy, or both. Thus, histologically normal proximal convolutions were more easily identified than were histologically abnormal proximal convolutions; this might lead the dissector, consciously or unconsciously, to dissect out the histologically normal tubules rather than the damaged tubules. In order to have a representative sample, every effort was made to dissect out for analysis both histologically normal and histologically abnormal tubules whenever there were distinct variations in the histological appearance of the tubules. Those tubules which were so abnormal that they could not be identified were analyzed as a separate group —"degenerated convolutions."

Levels of enzyme activity in renal tubular dysfunction. The results suggest that there are significant differences in enzyme activity between healthy kidneys and the nephrons in various disease states. Alkaline phosphatase activity was found to be decreased significantly in the convoluted tubules in all diseases studied, with the exception of cystinosis.

LDH activity was decreased significantly only in two of the three cases of adult Fanconi syndrome and also in the child with cystine storage disease and Fanconi syndrome. All three were excreting very large amounts of α-amino-nitrogen in the urine. The third adult with the Fanconi syndrome was excreting only a slight excess of α-amino-nitrogen; the LDH activity of the tubules was not decreased. These findings might suggest that the long continued massive amino acid depletion resulted in a decreased synthesis of enzyme protein in these patients. However, the finding of normal alkaline phosphatase activity in the tubules of the child with cystinosis makes this explanation unlikely. Therefore, alternative explanations must be sought.

In lupus glomerulonephritis, for example, where the tubular degeneration was of a comparatively mild degree, *increased* LDH activity was found in the convoluted tubules (10). There was no evidence of severe impairment of renal function in this disease, and glucose, inorganic phosphate, and amino acids appeared to be reabsorbed normally by the tubules. Thus far, *decreased* LDH activity has been found only in nephrons from the three kidneys with histologically severe tubular disorganization, and with severe derangements of tubular function; the lowest activity was observed in nephrons of the most severely damaged kidney. Thus the decreased LDH activity of these severely damaged nephrons may reflect or be a consequence of the severe damage sustained by the tubular cells in the basic disease processes of severe adult Fanconi syndrome and of cystine storage disease.

The *coefficient of variation* within individual kidneys was invariably larger than the coefficient of variation between individual kidneys (Table III), suggesting that the variation from nephron to nephron within a single healthy kidney was as great as, or greater than, the variation from healthy kidney to healthy kidney. There was little difference in the coefficient of variation for LDH activity in individual healthy and abnormal kidneys, suggesting that the nephron population of abnormal

TABLE III

Coefficients of variation for alkaline phosphatase and lactic dehydrogenase activities in healthy kidneys and in kidneys from patients with renal tubular dysfunction

	Alkaline phosphatase			Lactic dehydrogenase		
	Healthy kidneys		Renal tubular dysfunction	Healthy kidneys		Renal tubular dysfunction
Structure analyzed	Coefficient of variation within individuals*	Coefficient of variation between individuals	Coefficient of variation within individuals	Coefficient of variation within individuals	Coefficient of variation between individuals	Coefficient of variation within individuals
Glomeruli	65.3 (42.9–100)	35.4	75.4 (10.0–125.0)	31.9 (17.9–53.6)	20.4	22.4 (9.9–39.6)
Proximal convolutions	28.1 (16.4–36.0)	15.8	36.2 (11.6–74.1)	20.4 (8.4–34.4)	15.9	15.2 (4.8–24.8)
Distal convolutions	48.8 (28.1–65.2)	18.5	75.3 (34.3–179.0)	21.9 (12.0–36.9)	17.4	21.4 (10.7–48.0)

* Figures in parentheses indicate the range.

kidneys was as homogenous in this respect as was that of healthy kidneys. There was greater variation of alkaline phosphatase activity in individual abnormal than in individual healthy kidneys. This was due, at least in part, to the fact that the alkaline phosphatase activities were low in the abnormal kidneys, and the readings obtained were very close to those of the blanks (2).

Alkaline phosphatase and tubular reabsorption of glucose. It has been suggested by some investigators that alkaline phosphatase plays a significant role in the reabsorption of glucose from the glomerular filtrate by the proximal convolution (4, 5). If this were so, the alkaline phosphatase activity of the proximal convolution might be expected to be low in renal glycosuria. In the single patient with familial renal glycosuria studied, the alkaline phosphatase activity in the proximal convolution was decreased to approximately 50 per cent of that found in proximal convolutions from healthy kidneys. The decrease of alkaline phosphatase activity was of a similar magnitude in patients with lupus nephritis (10), none of whom had glycosuria. Moreover, in three patients with hypophosphatasia there was no glycosuria and the alkaline phosphatase activity was only 3.5 to 9.5 per cent of that found in healthy proximal convolutions. Unless an alternate pathway for glucose reabsorption can be invoked for patients with hypophosphatasia, these facts exclude the possibility that alkaline phosphatase plays a key role in the tubular reabsorption of glucose. To obtain further confirmation, glycosuria was produced in the dog by injection of phlorhizin. Table IV shows that there was no significant difference in the activity of this enzyme before and after injection of phlorhizin, so that phlorhizin-induced glycosuria is not mediated by way of inhibition of alkaline phosphatase.

TABLE IV

The effect of phlorhizin on the alkaline phosphatase and lactic dehydrogenase activity of the proximal convolutions in the dog *

Enzyme	Before phlorhizin	After phlorhizin	p
Alkaline phosphatase	20.6 ± 3.1 [9]	26.9 ± 16.3 [11]	0.29
Lactic dehydrogenase	215 ± 38 [10]	192 ± 28 [11]	0.67

* Total number of tissue fragments analyzed is given in brackets.

SUMMARY

1. Alkaline phosphatase and lactic dehydrogenase (LDH) activities were measured quantitatively in the various anatomical and functional parts of the nephrons from healthy kidneys and from the kidneys of six patients with diseases affecting the renal tubules: familial renal glycosuria, phosphate-losing renal tubular disease, cystine storage disease, and adult Fanconi syndrome.

2. Alkaline phosphatase activity was decreased in the convoluted tubules in all diseases studied except in cystine storage disease. LDH activity was decreased only in two of the three cases of

adult Fanconi syndrome and in a child with cystinosis and the Fanconi syndrome. A parallel decrease of both enzymes was observed in two of the patients with adult Fanconi syndrome.

3. The kidneys of three patients with hypophosphatasia were also studied. None had glycosuria. The extremely low level of alkaline phosphatase activity found in the proximal convolutions indicates that this enzyme does not play a key role in glucose reabsorption. This was confirmed by the observation that prolonged phlorhizin-induced glycosuria did not affect the level of alkaline phosphatase activity in the proximal convolution of the dog.

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QUANTITATIVE HISTOCHEMISTRY OF THE NEPHRON. V. ALKALINE PHOSPHATASE AND LACTIC DEHYDROGENASE ACTIVITIES IN LUPUS NEPHRITIS *

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The natural history and histological development of lupus nephritis have been studied in detail in our laboratory (1, 2). The glomeruli were more severely involved histologically than were the tubules at all stages of development of lupus nephritis. In the early stages (lupus glomerulitis) the histological abnormalities were confined to the glomeruli. Only when the glomerular involvement became considerably more severe was there histological evidence of tubular degeneration and interstitial fibrosis and inflammation (lupus glomerulonephritis). Even in lupus glomerulonephritis, histological abnormalities of the tubules and interstitial tissue were considerably less severe than in other types of glomerulonephritis. These histological observations were reflected functionally, in that ability to concentrate the urine and to excrete phenolsulfonphthalein were preserved until comparatively late in the disease.

In a previous paper of this series (3) the alkaline phosphatase and lactic dehydrogenase (LDH) activities of individual anatomical and functional units of the nephron were reported for healthy kidneys, and for a variety of diseases which affect primarily the renal tubule, i.e., diseases associated with a functional imbalance between tubules and glomeruli. In this paper, the results of assays of the activities of these two enzymes will be reported for lupus nephritis—a disease in which there appears to be histological and functional imbalance between glomeruli and tubules.

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MATERIAL AND METHODS

Clinical material

A total of 19 kidney specimens was analyzed for alkaline phosphatase and LDH activity. Eighteen specimens were taken by percutaneous renal biopsy (4), and 1 specimen was obtained at autopsy. Nine specimens were analyzed on 4 patients, studied serially, and a biopsy and autopsy specimen were taken from 1 patient.

An independent and detailed histological analysis was made on each specimen according to the methods previously described (1). On the basis of this analysis the specimens assayed for enzyme activity were divided into the following groups.

1. Lupus glomerulitis (5 biopsies). This term was used to indicate a proliferative and/or membranous lesion of the glomerular tufts in the absence of significant tubular damage or changes in the interstitial tissue (1). The glomeruli were involved to a mild degree in all five.

2. Lupus glomerulonephritis (12 biopsies). This term was used to indicate a more severe membranous and/or proliferative lesion of the glomeruli, associated with definite tubular and interstitial tissue changes (1). The kidneys with lupus glomerulonephritis were subdivided into two groups as shown below.

a) Active lupus glomerulonephritis (5 biopsies). Definite morphologic evidence of activity was observed in the glomerular lesions, i.e., one or more of the following histological features was prominent: local necrosis, karyorrhexis, hematoxyphil bodies, fibrinoid "hyaline" thrombi, and "wire loops" (1, 2). Two of the five patients were receiving prednisone (15 and 20 mg daily) when the biopsy was taken; the other three had not been treated.

b) Healed or healing lupus glomerulonephritis (7 biopsies). Thickening of the glomerular basement membrane was the predominant lesion found in these biopsies, all or most of the active lesions present in previous biopsies from the same patients having disappeared. The abnormalities found in the tubules and interstitial tissue were similar to or less severe than those found in active lupus glomerulonephritis (2). All the patients had been treated with large doses of prednisone (40 to 60 mg

daily) for at least 6 months before the renal biopsies were taken (2).

One biopsy and an autopsy specimen from a single patient were considered separately because, in addition to healing lupus glomerulonephritis, there was partial occlusion of both renal arteries with consequent ischemic changes in the kidneys.

Enzyme assays

The techniques for handling the tissues and for dissection and weighing of the individual units of the nephron have been described (5). The dissected fragments were assayed for alkaline phosphatase activity and for LDH activity, under conditions found to be optimal for the human kidney (5, 6). Results were expressed in moles of substrate split or moles of DPNH oxidized per hour at 37° C per kilogram dry weight of tissue (MKH units).

Glomerular weight and density

In the course of dissecting and weighing glomeruli for enzyme assay we observed that glomeruli from kidneys with lupus nephritis appeared to be both larger and heavier than glomeruli from normal kidneys. A statistical comparison was therefore made of the weights recorded on 130 glomeruli from normal adult kidneys and 234 glomeruli from kidneys involved with lupus nephritis. In addition, the diameters of the glomeruli from each specimen were measured on the stained control sections, using a microscope with a projection viewer. Two measurements were made at right angles to each other on each glomerulus, to obtain an average diameter. Ten glomeruli from each kidney were measured to give an average glomerular diameter for each kidney. The segments of glomeruli which were weighed had been dissected out from 16 μ sections and all were spherical segments close to the meridians of the tufts. The polar segments and those near the poles were specifically excluded (the terms "pole" and "meridian" refer to the plane of the section). The ratio of glomerular weight to the square of glomerular diameter was calculated as an *index* of the density of the glomeruli.

RESULTS

The results of assays for alkaline phosphatase and LDH activities are given in Tables I and II.

Alkaline phosphatase activity was within normal limits in the glomeruli of patients with lupus nephritis (Table I). However, the alkaline phosphatase activity was considerably decreased in the proximal convolutions ($t = 7.3$, $p = < 0.001$) and in the distal convolutions ($t = 6.36$, $p = < 0.001$). These differences were found both in lupus glomerulonephritis (active and healing) and in lupus glomerulitis, in which the tubules were normal histologically.

LDH activity in lupus nephritis was not significantly different from that found in healthy kidneys (Table II), except in the case of the proximal convolutions in which the activity was slightly higher ($t = 2.82$, $p = < 0.02$). However,

TABLE I

Alkaline phosphatase activity in the anatomical units of nephrons from healthy kidneys and from kidneys of patients with lupus nephritis (expressed in moles of substrate split per kilogram dry weight of tissue per hour at 37° C) *

Structure analyzed	Healthy adult kidneys 6†	Lupus glomerulitis 2	Lupus glomerulonephritis: Active 5	Lupus glomerulonephritis: Healing 7	All lupus nephritis 14
Glomeruli	0.48±0.17 *5* [36]	0.46±0.15 *2* [13]	0.50±0.27 *5* [27]	0.54±0.31 *7* [41]	0.51±0.25 *14* [81]
Proximal convolutions	5.70±0.90 *5* [40]	1.98±0.33 *2* [16]	2.41±0.62 *5* [46]	2.61±0.89 *7* [62]	2.45±0.72 *14* [124]
Distal convolutions	2.70±0.50 *6* [40]	1.05±0.48 *2* [12]	1.05±0.69 *5* [21]	1.17±0.55 *7* [35]	1.11±0.54 *14* [68]
Degenerated convolutions			4.80 *1* [4]	2.12±0.65 *2* [11]	3.01±1.62 *3* [15]
Medullary rays	2.84 *1* [9]	1.15±0.08 *2* [14]	1.05±0.94 *2* [11]	2.19±1.02 *6* [28]	1.75±1.01 *10* [53]
Medulla (outer and inner medullary zones)	0.80±0.20 *2* [11]		0.50±0.13 *2* [24]	0.94±0.45 *2* [31]	0.72±0.37 *4* [55]
Papilla	1.70±0.89 *2* [15]		0.44 *1* [7]	0.77 *1* [7]	0.61±0.21 *2* [14]
Small artery	1.20±0.70 *5* [25]	0.80 *1* [1]	0.61±0.48 *4* [16]	0.42±0.26 *6* [31]	0.52±0.35 *11* [48]

* Mean values and standard deviations are given for the averages of each kidney analyzed. Total number of tissue fragments analyzed is given in brackets.

† Number of kidneys analyzed in each group is shown in italics.

TABLE II

Lactic dehydrogenase activity in the anatomical units of nephrons from healthy kidneys and from kidneys of patients with lupus nephritis (expressed in moles of DPNH oxidized per kilogram dry weight of tissue per hour at 37° C) *

Structure analyzed	Healthy adult kidneys 7†	Lupus glomerulitis 4	Lupus glomerulonephritis		All lupus nephritis 14
			Active 4	Healing 7	
Glomeruli	108 ± 22 6 [46]	84 ± 12 4 [26]	129 ± 22 4 [22]	136 ± 38 7 [41]	120 ± 37 15 [89]
Proximal convolutions	257 ± 41 7 [55]	207 ± 32 4 [35]	352 ± 30 4 [26]	364 ± 78 7 [48]	319 ± 86 15 [109]
Distal convolutions	256 ± 44 7 [49]	225 ± 62 4 [27]	338 ± 63 4 [20]	374 ± 128 6 [40]	321 ± 114 14 [87]
Degenerated convolutions			294 ± 30 2 [9]	313 ± 167 4 [24]	306 ± 162 6 [33]
Medullary rays	231 ± 25 3 [25]	204 ± 36 4 [27]	312 ± 34 2 [13]	320 ± 101 5 [32]	276 ± 90 11 [72]
Medulla (outer and inner medullary zones)	165 ± 21 3 [22]		212 1 [12]	239 ± 12 2 [29]	230 ± 17 3 [41]
Papilla	175 ± 82 3 [8]		130 1 [5]	225 1 [6]	178 ± 67 2 [11]
Small artery	131 ± 61 6 [31]	45 ± 38 3 [20]	171 ± 13 2 [11]	100 ± 48 6 [30]	98 ± 58 11 [61]

* Mean values and standard deviations are given for the averages of each kidney analyzed. Total number of tissue fragments analyzed is given in brackets.

† Number of kidneys analyzed in each group is shown in italics.

significant differences were evident when the kidneys with lupus nephritis were subdivided, on the basis of the histology, into lupus glomerulitis and lupus glomerulonephritis. In lupus glomerulitis the LDH activity was slightly, but not significantly, less than that found in healthy kidneys. However, the LDH activity of proximal convolutions was increased significantly above normal, both in active ($t = 4.4$, $p = < 0.01$) and in healing lupus glomerulonephritis ($t = 3.22$, $p = < 0.01$). The activity in the distal convolutions was increased slightly above normal, but the differences were of questionable significance (active, $t = 2.31$, $p = < 0.05$; healing, $t = 2.15$, $p = 0.05$). No differences were observed between the LDH activity in the nephron in active and in healing lupus glomerulonephritis.

From these data it will be noted that there were significant differences between the enzyme activities in lupus glomerulitis and lupus glomerulonephritis, and this was found for all units of the nephron analyzed. There was virtually no overlap between the activities found in these two groups (glomeruli, $t = 4.26$, $p = < 0.001$; proximal convolutions, $t = 6.34$, $p = < 0.001$; distal convolutions, $t = 3.04$, $p = < 0.02$; medullary rays, $t = 3.16$, $p = < 0.02$). An analysis of variance (7) was also done in order to assess the significance of the variations within the three subgroups of lupus nephritis. This showed that the differences between the mean activities for the three groups, taking into account the individual variation within each group, were significant in the case of the glomeruli ($F = 4.19$, $p = < 0.05$), highly significant in the case of the proximal convolutions ($F = 11.2$, $p = < 0.01$), but not significant for the distal convolutions ($F = 2.85$, $p = > 0.05$).

Effect of ischemia on enzyme activity. The data from one patient with healing lupus glomerulonephritis, studied on two occasions, are not included in Tables I and II. In addition to lupus nephritis an old thrombosis was present in the aorta of this patient, extending to the bifurcation and partially occluding the renal arteries. Histologically, there was evidence of patchy, severe ischemic changes in addition to the lesions of lupus glomerulonephritis. The enzyme activities in the glomeruli and convoluted tubules of this patient are given in Table III. In the nonischemic nephrons the levels of activity of both enzymes were comparable with those found in other cases of lupus glomerulonephritis, whereas extremely low enzyme activities were found in severely ischemic nephrons.

TABLE III

Results of assay of alkaline phosphatase and lactic dehydrogenase activities in the nephrons from a patient with healing lupus glomerulonephritis and severe vascular ischemia (expressed in moles of substrate split per kilogram dry weight of tissue per hour at 37° C) *

Structure analyzed	Alkaline phosphatase			Lactic dehydrogenase		
	Severely ischemic nephrons	Completely hyalinized nephrons	Nonischemic nephrons	Severely ischemic nephrons	Completely hyalinized nephrons	Nonischemic nephrons
Glomeruli	0.18 ± 0.07 [4]	0.14 ± 0.05 [11]	0.45 ± 0.38 [2]	33.6 ± 13.8 [5]	8.03 ± 1.71 [6]	132 ± 10.3 [4]
Convoluted tubules	0.48 ± 0.23 [16]	0.34 ± 0.105 [6]	3.26 ± 0.29 [8]	60.0 ± 9.1 [15]		250 ± 16.1 [12]

* Results are expressed as means with standard error of the means, and the number of fragments analyzed is in brackets.

Variability of the observations. The coefficients of variation for alkaline phosphatase and LDH activities are shown in Table IV. The coefficient of variation *between* healthy individuals was smaller than that *within* healthy individuals, an observation which was also made in studies on normal animals (5, 6). The same was true for lupus nephritis. The variation of alkaline phosphatase activity within individuals with lupus nephritis was greater than within healthy kidneys. The variation in LDH activity from nephron to nephron was similar to that found in the normal. The average variation from kidney to kidney within each histological subgroup of lupus nephritis was somewhat greater in the case of alkaline phosphatase activity than the variation from healthy kidney to healthy kidney. No such difference was found in the case of LDH activity.

Effect of prednisone on enzyme activity. Most of the renal biopsies assayed were taken from patients who were being treated with prednisone. The patients with healing lupus glomerulonephritis were receiving an average dose of 50 mg prednisone daily, whereas most of those with lupus glomerulitis and with active lupus glomerulonephritis had not been treated with steroid hormones or drugs, or were being treated with lower doses of prednisone (approximately 15 to 20 mg per day). In order to examine the effects of prednisone on the enzyme activity of the nephron, an experiment was done on rats. Renal biopsies were taken from four adult Sprague-Dawley rats, weighing an average of 327 g. Each rat was then injected intramuscularly with prednisolone, 1 mg daily, for 14 days, and was then sacrificed. Enzyme assays were made on the glomeruli and on the proximal and distal convolutions of the tubules from the biopsy and autopsy specimens of the same rat. The results are shown in Table V. It is evident that prednisolone in this dosage—

TABLE IV

Coefficient of variation for alkaline phosphatase and lactic dehydrogenase activity in healthy kidneys and in kidneys from patients with lupus nephritis

Structure analyzed	Alkaline phosphatase				Lactic dehydrogenase			
	Healthy kidneys		Lupus nephritis		Healthy kidneys		Lupus nephritis	
	Coefficient of variation within individuals*	Coefficient of variation between individuals	Coefficient of variation within individuals	Coefficient of variation between individuals within each group	Coefficient of variation within individuals	Coefficient of variation between individuals	Coefficient of variation within individuals	Coefficient of variation between individuals within each group
Glomeruli	65.3 (42.9–100)	35.4	76.6 (18–165)	48.3	31.9 (17.9–53.6)	20.4	21.5 (6–48)	19.7
Proximal convolutions	28.1 (16.4–36.0)	15.8	48.3 (13–92)	25.6	20.4 (8.4–34.4)	15.9	18.6 (8–34)	15.3
Distal convolutions	48.8 (28.1–65.2)	18.5	62.1 (23–113)	53.0	21.9 (12.0–36.9)	17.4	27.3 (12–46)	26.7

* The range is given in parentheses.

TABLE V

The effect of prednisolone, 1 mg daily for 14 days, on alkaline phosphatase and lactic dehydrogenase activity in the individual anatomical units of nephrons of the rat *

Structure analyzed	Rat 1		Rat 2		Rat 3		Rat 4	
	Before prednisolone	After prednisolone	Before prednisolone	After prednisolone	Before prednisolone	After prednisolone	Before prednisolone	After prednisolone
				Alkaline phosphatase				
Glomeruli	2.66±0.56 [7]	2.41±0.11 [6]	5.12±0.81 [8]	3.98±1.08 [11]	1.54±0.40 [6]	2.04±0.23 [6]	4.02±0.89 [6]	3.70±0.69 [5]
Proximal convolutions	21.6±1.87 [13]	15.8±1.46 [7]	24.2±2.09 [10]	21.6±1.72 [6]	8.11±0.91 [7]	9.47±1.10 [6]	19.7±1.16 [7]	23.3±2.84 [8]
Distal convolutions	1.51±0.51 [6]	2.39±0.42 [7]	1.86±0.61 [12]	1.74±0.52 [13]	1.52±0.29 [5]	1.78±0.40 [7]	1.81±1.24 [7]	2.61±0.75 [7]
				Lactic dehydrogenase				
Glomeruli	76.7±3.27 [6]	83.5±6.50 [6]	77.3±4.78 [6]	70.8±11.7 [5]	78.0±12.1 [7]	78.0±0.74 [6]	77.3±8.45 [7]	71.5±19.5 [5]
Proximal convolutions	128±12.2 [12]	115±6.44 [12]	160±8.45 [7]	137±14.0 [5]	119±4.65 [7]	134±7.94 [6]	112±13.7 [7]	132±13.5 [6]
Distal convolutions	364±36.2 [5]	268±35.4 [7]	350±36.4 [6]	342±21.8 [6]	297±7.96 [5]	389±16.8 [7]	330±26.2 [7]	285±52.3 [5]

* Results, expressed in MKH units, are means with standard errors. The number of fragments of tissue analyzed is given in brackets.

which, on a weight for weight basis, is approximately four times that used in the patients—had no significant or consistent effect on the enzyme activity of the glomeruli or convoluted tubules.

The weights, diameters and derived densities of the glomeruli from healthy kidneys and from kidneys with lupus nephritis are given in Table VI. The impression that glomeruli of lupus nephritis were heavier than glomeruli from healthy kidneys was confirmed; they were 40 per cent heavier than those of healthy kidneys and the difference was significant statistically ($t = 3.6$, $p = < 0.01$). The heaviest glomeruli were found in the group with active lupus glomerulonephritis. The diameter of the glomeruli was increased significantly only in the group with active lupus glomerulonephritis ($t = 3.28$, $p = < 0.01$). The density index of the glomeruli

$$\left(\frac{\text{glomerular weight (millimicrograms)}}{\text{glomerular diameter (microns)}^2} \times 10^3\right)$$

was somewhat increased in lupus nephritis, as compared to the normal, but this difference was not statistically significant ($t = 2.01$, $p = > 0.05$). The greatest density was found in healing lupus glomerulonephritis.

TABLE VI

The weights, diameters and density of glomeruli from healthy kidneys and from kidneys of patients with lupus nephritis *

	Healthy kidneys	Lupus glomerulitis	Lupus glomerulonephritis		All lupus nephritis
			Active	Healing	
Glomerular weight (mμg)	42.1 ± 7.81 *9*† [130]	56.8 ±13.82 *5* [51]	66.5 ±18.62 *5* [82]	55.9 ±17.30 *7* [101]	59.3 ±16.49 *17* [234]
Glomerular diameter (μ)	179.8 ±16.4 *9* [90]	196.0 ±27.3 *5* [50]	204.5 ±11.6 *5* [50]	179.6 ±10.6 *7* [70]	191.7 ±19.7 *17* [170]
Glomerular density‡	1.32± 0.29 *9*	1.57± 0.66 *5*	1.59± 0.41 *5*	1.75± 0.65 *7*	1.65± 0.55 *17*

* Values are expressed as means with standard deviations of the means for each kidney. Total number of glomeruli analyzed is given in brackets.

† Number of kidneys analyzed is given in italic type.

‡ The density index of the glomeruli = $\frac{\text{glomerular weight}}{\text{glomerular diameter}^2} \times 10^3$.

DISCUSSION

Alkaline phosphatase activity was found to be decreased in the proximal and distal convolutions of the tubules in patients with lupus nephritis, but the level of enzyme activity was not related to the presence or severity of morphologic changes in the tubules. The alkaline phosphatase activity of the glomeruli was within normal limits, and there were no apparent effects of glomerular damage of varying severity on the level of enzyme activity found. The coefficient of variation of alkaline phosphatase activity from nephron to nephron in the same kidney was greater for lupus nephritis than for healthy kidneys. However, this was almost certainly due, at least in part, to technical artifact, particularly in the abnormal kidneys analyzed, for alkaline phosphatase activities in them were close to those of the blanks (3, 5).

By contrast, LDH activity was decreased slightly, but not to a significant degree, only in lupus glomerulitis; whereas there was a slight but statistically significant increase in the activity of this enzyme in the glomeruli and throughout the tubules in lupus glomerulonephritis. The differences between enzyme activity in lupus glomerulitis and lupus glomerulonephritis were statistically significant in the glomeruli and proximal convolutions of the tubules. Thus, there was an inverse relationship between LDH activity and histological evidence of degeneration of the proximal and distal convolutions.

These differences were not related to the presence or degree of renal functional impairment: in lupus glomerulonephritis the average specific gravity concentration was 1.020; the average creatinine clearance was 76 ml per minute; the average urea clearance was 56 per cent of average normal renal function; and the average phenolsulfonphthalein excretion in 15 minutes was 28 per cent. None of these patients excreted excess glucose, phosphate or amino acids in the urine.

The differences in LDH activity did not appear to be related to the dosage of prednisone used to treat these patients. The patients with healing lupus glomerulonephritis were receiving large doses of prednisone, whereas both those with lupus glomerulitis and those with active lupus glomerulonephritis were untreated, or were being treated with small doses of prednisone. Moreover, no significant or consistent effect on enzyme activity was observed when prednisolone was injected into rats.

Glomerular damage in lupus nephritis precedes the appearance of significant structural changes in the convoluted tubules. The morphologic changes in the glomeruli may be very severe before definite tubular damage is evident—in this respect, lupus nephritis differs from many other renal diseases such as glomerulonephritis and pyelonephritis. This could result in a physiological situation in which the renal tubules, particularly the proximal convolutions, perform increased work in reabsorbing from the tubular lumens substances such as protein filtered in excess by the glomeruli, or in excreting other substances into the tubular lumens. Thus, the relative increase in LDH activity in lupus glomerulonephritis might be an enzymatic expression of the preserved function of these tubules and of their ability to respond metabolically to increased metabolic demands. By contrast, in primary renal tubular diseases, the LDH activity of the convoluted tubules was normal in the presence of mild tubular abnormalities and was significantly decreased when the tubules were severely damaged (3). The view that the tubules in lupus glomerulonephritis are capable of responding to increased metabolic demands would be consistent with the hypothesis of Platt, namely, that in chronic renal disease residual functioning nephrons may be capable of normal activities and even of functional adaptations which may result in an increase of work done per residual functioning nephron (8). The view of Platt has gained considerable support from the studies of Bricker, Morrin and Kime on the adaptive changes in residual nephrons of dogs with experimenal unilateral renal disease (9). Their investigations strongly suggest that the surviving nephrons in diseased kidneys function normally, and that specific functional sites are not selectively destroyed in different nephrons, a condition which would result in great functional differences from nephron to nephron.

In our studies the coefficient of variation of LDH activity from nephron to nephron in the same kidney was similar for healthy kidneys and for lupus nephritis (Table IV), suggesting that the nephron population examined in lupus nephritis was as homogenous with respect to LDH ac-

tivity as was the nephron population of healthy kidneys. The only exception to this was in the case of grossly ischemic and probably nonfunctioning nephrons (Table III) in which the LDH activity was extremely low.

The relationship between the histology of the glomeruli and their weight, diameter and density. In lupus nephritis fibrinoid and hyaline materials are deposited on the endothelial surface of the glomerular basement membrane, and there is frequently an increase of both endothelial and epithelial cells. A wide variety of other changes also occurs in the glomeruli (2). The finding that the weight of glomeruli was 40 per cent greater in lupus nephritis than in healthy kidneys was consistent with these histological observations. The most severe and most widespread histological changes were seen in active lupus glomerulonephritis, in which condition the glomeruli were heaviest, and were found to have the greatest diameter. The densest glomeruli, however, were found in healing lupus glomerulonephritis. In healing lupus glomerulonephritis the active glomerular lesions had almost entirely disappeared, leaving glomeruli which had a diffusely thickened basement membrane as the major lesion. These glomeruli did not look large histologically, and their diameter was similar to that of healthy glomeruli, but their density was greater than that of healthy glomeruli and of glomeruli in both the other groups of lupus nephritis. This was consistent with the finding of diffuse capillary basement membrane thickening.

SUMMARY

1. Alkaline phosphatase and lactic dehydrogenase (LDH) activities were measured in the individual anatomic units of the nephron in 18 biopsy and one autopsy specimen from 13 patients with lupus nephritis.

2. Alkaline phosphatase activity was considerably less than normal in the proximal and distal convolutions of the tubules in lupus glomerulitis and in lupus glomerulonephritis.

3. LDH activity was increased in lupus glomerulonephritis but not in lupus glomerulitis. The variation of LDH activity from nephron to nephron was no greater in lupus nephritis than in healthy kidneys. In this respect the diseased nephrons were as homogenous as healthy nephrons.

4. The weights of 130 glomeruli from healthy kidneys and of 234 glomeruli from kidneys affected by lupus nephritis were measured. The glomeruli in lupus nephritis were found to be 40 per cent heavier than those from healthy kidneys, and scarred, healed glomeruli were found to be denser than those in which the disease was active.

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VARIATIONS IN THE PULMONARY CAPILLARY BLOOD VOLUME AND MEMBRANE DIFFUSION COMPONENT IN HEALTH AND DISEASE *

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Forster, Roughton and their colleagues (1, 2) have reported a method of subdividing the components of the pulmonary diffusing capacity by measurement of the apparent diffusing capacity at different alveolar oxygen tensions. This development followed preparatory work by the same authors (3, 4) in which the kinetic factors involved in CO and hemoglobin combination were reported. The principles underlying the measurements have been fully discussed by Forster in a recent review (5).

Lewis, Lin, Noe and Komisaruk (6) reported on the measurement of pulmonary capillary blood volume in normal subjects, using the single breath method of determining the pulmonary diffusing capacity. They found average values of 65 ml for the pulmonary capillary blood volume at rest and 98 ml CO per minute per mm Hg for the membrane diffusion component. In four subjects both of these quantities increased somewhat during exercise. They did not report any results on patients. McNeill, Rankin and Forster (7), using a similar technique of measuring the pulmonary diffusing capacity by a single breath method, found similar values at rest for the pulmonary capillary blood volume, but a lower value for the membrane component, which was about 63.5 ml CO per minute per mm Hg in normal subjects at rest. These authors reported the results of this measurement in five patients with various types of pulmonary fibrosis, two with pulmonary hypertension, two with pulmonary congestion, and three with chronic obstructive emphysema. The most significant findings were the decrease in membrane diffusion component in patients with pulmonary fibrosis, and the increase generally found in pulmonary capillary blood volume in patients with increased pressure in the lesser circulation.

The present study reports the measurement of these two components of the pulmonary diffusing capacity by a steady state method during exercise in 14 normal subjects and in a group of 22 patients. This work was undertaken to study the reliability of the results obtainable with this technique, and to explore its usefulness in investigative work.

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METHODS

The apparatus used in this work has recently been described in full (8). A steady state measurement of diffusing capacity (D_L) is made, the mean alveolar CO tension being calculated from an assumed value of respiratory dead space. Technical details and a study of the errors of this method have been presented previously (8). The determination of pulmonary capillary blood volume (V_c) and membrane diffusion component (D_M) involves the measurement of the pulmonary diffusing capacity at two different oxygen tensions. Each experiment whether on a normal subject or a patient consisted in the measurement of the diffusing capacity on either 60 or 100 per cent oxygen, followed by its determination on air. All estimates were made during moderate or light exercise.

In a typical determination, the subject attained a steady state on the exercise treadmill for 5 minutes, and was then switched into either 60 or 100 per cent oxygen for a further period of about 5 minutes. At the end of this time, without interruption of the exercise, he was connected to the inspiratory bag which contained the same oxygen concentration as he had been breathing, with the addition of approximately 0.20 per cent CO. The inspired and expired gases were monitored in the manner described previously, and a careful note was made of the total time during which the subject breathed CO. The subject then rested on a chair, and during this time a sample of venous blood was drawn for the measurement of carboxyhemoglobin (COHb) by the technique of Lawther and Apthorp (9). With the treadmill in the same position and at the same speed, the subject then resumed his exercise, the sequence of events being timed as before, but on this occasion he was switched into a mixture of air containing about 0.12 per cent CO. Following the determinations of the inspired and mixed expired CO concentrations, the oxygen uptake was calculated using the monitored mixed expired CO_2 and oxygen in the manner previously described (8). The total experiment

with the two measurements of diffusing capacity took about 25 minutes. Values for D_L at each oxygen tension were not "pooled"; each experiment consisted in the double determination of D_L followed by the calculation of V_c and D_M values from these two points. This procedure was followed so that the standard deviation of repeat determinations would provide an indication of the likely error of a single determination of V_c and D_M based on one determination of D_L at each oxygen tension. A higher inspired CO concentration was used during the 100 per cent oxygen determination to lower the proportionate effect of "back pressure" of CO in relation to alveolar CO concentration.

CALCULATIONS

The pulmonary diffusing capacity was calculated from the $D_{CO_{1A}}$ as previously described (8) except that the COHb correction was slightly modified. Early experience with the method showed that the calculated values of V_c and D_M were very sensitive to this correction, and it was found necessary to calculate the mean COHb during the exercise run, rather than to use the COHb level measured at the end of exercise as the pertinent value. This change alters the calculated D_L by not more than one unit, but this is sufficient to affect the calculated V_c.

The corrected D_L was calculated as previously described by Linderholm's method (10). The circulating blood volume of the subject was calculated from the formulas: 32.18 cc per lb in males and 32.14 cc per lb in females.

The alveolar oxygen tension ($P_{A_{O_2}}$) was calculated from the Bohr alveolar gas equation using a mixed expired oxygen percentage of $(100 - F_{E_{CO_2}})$ or $(60 - F_{E_{CO_2}})$ depending on which oxygen mixture had been used. During the air diffusing capacity determination the measured mixed expired oxygen concentration was used in this calculation. Previous work from this laboratory (8) has supported the view of Asmussen and Neilsen (11) that the alveolar oxygen so calculated during exercise in normal subjects is reasonably accurate. The mean capillary oxygen tension ($\bar{P}_{C_{O_2}}$) was then calculated by the method described by Linderholm (10). A "λ value" of 2.5 (2, 3, 5) was assumed for all subjects. The function $1/\theta$ was calculated from the formula,

$$1/\theta = [(0.0057 \times \bar{P}_{C_{O_2}})] + 0.75,$$

which was derived from Figure 1 (2).

The pulmonary midcapacity during exercise was calculated by adding half the tidal volume to the measured resting functional residual capacity (FRC), a technique shown to give a reasonably accurate estimate of this figure during exercise (8).

The V_c and the D_M were then calculated by solving the equation $1/D_{L_{CO}} = 1/\theta V_c + 1/D_M$ for the two determinations of pulmonary diffusing capacity at the two oxygen tensions. It will be appreciated that this calculation necessarily involves the assumption that the switch

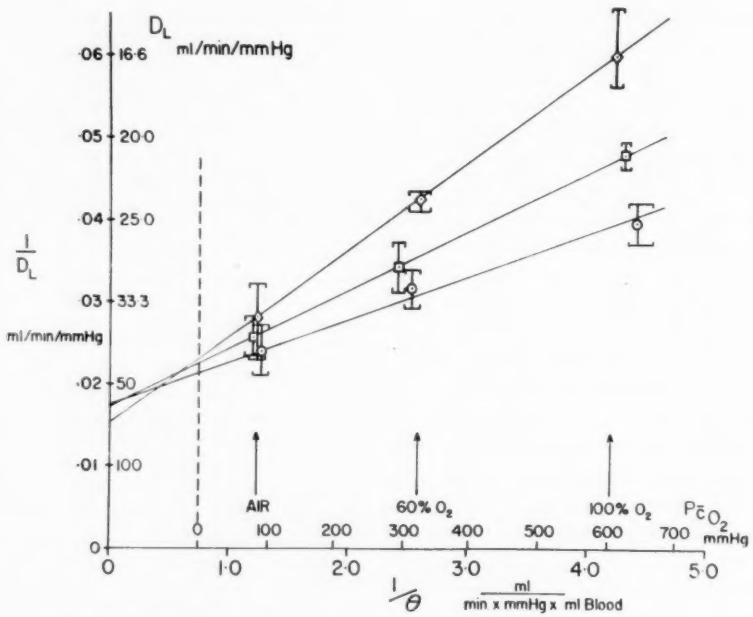

FIG. 1. RELATIONSHIP BETWEEN $1/D_L$ AND MEAN ALVEOLAR CAPILLARY OXYGEN TENSION ($\bar{P}_{C_{O_2}}$) IN SUBJECTS RED ⊡, CJV ⊙, and DVB ◈. The lines about the mean values represent the actual ranges of the observation of D_L and $\bar{P}_{C_{O_2}}$ in these subjects, all of whom were exercising at 3 mph on a flat surface.

from air to oxygen has not changed the pulmonary capillary bed significantly, nor altered the nature or area of the pulmonary membrane.

Very accurate determinations of D_L are required if variability in the calculated pulmonary capillary blood volumes and membrane components is to be avoided. An important precaution is the avoidance of contaminated CO mixtures as mentioned previously (8), and accuracy in the determination of COHb. The correction due to this factor becomes considerable if a heavy smoker is studied, and is more significant when he is breathing oxygen than air. We have found that the calculated back tension of CO must not be allowed to exceed more than 15 per cent of the alveolar CO pressure if considerable errors in the determined value of V_c are to be avoided. When heavy smokers are studied, the inspired CO percentage may have to be increased to 0.30 per cent during the 100 per cent oxygen determination to keep the calculated back CO tension less than 15 per cent of the alveolar CO tension.

RESULTS

Normal subjects. The results are shown in Tables I through VIII, and in Figure 1. In Table I are shown the physical characteristics of the 14 normal subjects studied, together with the results of resting pulmonary function tests. The ages of these subjects varied from 24 to 46. The resting pulmonary function tests were all within predicted limits. In Table II are shown the results of determinations of pulmonary capillary blood volumes and membrane diffusion components in four of these subjects. At a speed of 3 mph with the treadmill flat, a total of 20 determinations was made on these four subjects, all on different days, and four observations were made on Subject DVB at 3 mph on a 10 per cent treadmill grade. The ranges of variation together with the mean values are shown in this table and below each column are the standard deviations of the observations. The extreme right-hand column of this table shows the number of estimations done on 100 or 60 per cent oxygen on each individual. The most variable results were in the determination of V_c in Subject RED. One determination was double another at approximately the same oxygen uptake and this led to the finding of a high standard deviation for the five observations recorded. If this single result be omitted, the standard deviations are less than $\pm$ 12.5 for the determination of V_c on these four subjects. It is clear from the data that there are significant differences in the mean values of V_c between different normal subjects, those, for instance, in LSB being uniformly and significantly lower than those in CJV or RED. There is less variability and rather less variation between individuals in the measured D_M, and the differences in this component disappear when it is related to lung volume (D_M/MC). The V_c is still significantly different after this correction, however (V_c/MC). In Subject DVB, an increase in exercise resulted in a significant increase in the over-all D_L on air (D_L/air), and in V_c, but there was no change in D_M. This suggests that the increased D_L on exercise is mainly accounted for by an increase in V_c, though this conclusion might not necessarily be applicable to the transition from rest to exercise.

In Table III, the subjects who are below 35

TABLE I

*Physical data and results of resting pulmonary function tests in normal subjects**

No.	Subject	Sex	Age	Ht	Wt	VC	FRC	ME%	MMFR
				in	*lbs*	*L*	*L*		*L/sec*
1	RED	M	30	70½	165	4.97	3.18	67	3.92
2	CJV	M	29	71½	195	5.04	2.92	78	4.60
3	FM	M	28	74	205	5.77	3.77	60	6.80
4	GW	M	34	69½	168	4.91	3.54	66	5.90
5	DP	M	24	72½	182	5.04	3.60	71	5.00
6	RF	M	25	65	137	4.40	2.28	71	6.00
7	JE	M	31	71	186	(4.10)	(3.20)		
8	DVB	M	37	70	180	3.33	2.75	92	3.41
9	LSB	M	41	66½	168	3.00	3.26	58	3.50
10	REF	M	39	74	165	(4.40)	(3.50)		
11	WF	M	44	68	163	4.45	2.91	52	2.80
12	JAP	M	42	75	193	4.00	3.21	58	3.90
13	REGP	M	46	66½	173	3.58	1.72	53	3.00
14	JB	M	45	72	211½	4.95	3.02	91	7.00

* VC = vital capacity; FRC = functional residual capacity; ME % = closed circuit helium mixing index; MMFR = maximal midexpiratory flow rate. Results shown in parentheses have been predicted from the subject's height.

TABLE II

*Results of repeated estimates of pulmonary capillary blood volume (V_c) and membrane diffusion component (D_M) in four normal subjects**

Subject	Rate	Grade	Minute vol	$\dot{V}_{O_2}$	$\dot{V}_{O_2}/\dot{V}_{air}$	D_{LCO} (air)	V_c	D_M	D_{LCO}/MC	V_c/MC	D_M/MC	No. of estimations
	mph		*l/min*	*l/min*			*ml*					
DVB												
Mean†	3	10%	43.6	1.68	3.42	39.3	122	62.0	9.62	29.6	15.3	100% O_2 × 3
			49.2	1.90	3.86	41.0	131	64.2	10.1	32.3	15.8	60% O_2 × 1
			53.5	2.22	4.17	43.9	135	68.0	11.0	33.9	16.4	Total = 4
SD ±			3.6	0.19	0.31	1.34	5.25	2.36	0.53	1.64	0.5	
			25.7	1.10	3.85	32.5	81	52.2	8.6	20.1	14.5	100% O_2 × 3
Mean	3	Flat	28.4	1.21	4.26	35.3	96	66.5	9.2	25.3	17.4	60% O_2 × 3
			30.2	1.29	4.63	38.6	109	87.0	9.91	30.3	21.5	Total = 6
SD ±			1.45	0.07	0.26	2.34	11.1	11.4	0.40	3.39	2.5	
RED												
			22.8	1.13	5.37	35.3	122	45.3	9.21	31.3	11.9	100% O_2 × 2
Mean	3	Flat	25.3	1.21	4.79	38.8	163	58.2	10.0	42.0	15.0	60% O_2 × 3
			27.4	1.33	4.17	44.0	261	75.7	11.10	67.5	19.1	Total = 5
SD ±			1.53	0.08	0.43	3.0	50.4	11.8	0.65	13.2	2.84	
CJV												
			28.1	1.21	3.17	37.0	182	47.0	9.05	46.8	11.6	100% O_2 × 3
Mean	3	Flat	31.7	1.27	4.01	41.5	200	56.8	10.3	49.4	14.0	60% O_2 × 2
			39.1	1.38	4.42	46.8	214	67.2	11.5	52.7	16.5	Total = 5
SD ±			3.82	0.06	0.46	4.2	12.5	8.1	1.04	2.2	2.1	
LSB												
			21.1	0.98	4.02	20.2	58.0	38.5	6.85	19.7	13.1	100% O_2 × 2
Mean	3	Flat	23.7	1.04	4.39	22.1	68	40.2	7.5	22.9	13.9	60% O_2 × 2
			24.9	1.14	4.80	23.9	80.2	43.1	7.96	26.6	14.4	Total = 4
SD ±			1.21	0.06	0.30	1.6	8.45	1.55	0.43	24.8	5.2	

* Columns 9, 10 and 11 show the values given in the preceding three columns divided by the exercise midcapacity in liters. The final column shows the number of estimates done on air and 100 per cent oxygen, or on air and 60 per cent oxygen on each subject. $\dot{V}_{O_2}$ = oxygen consumption; $\dot{V}_{O_2}/\dot{V}_{air}$ = oxygen consumption per liter ventilation; D_{LCO} = diffusing capacity of the lung for CO; MC = ventilated lung volume.
† Figures above and below the mean value indicate the range of observations.

years of age have been grouped together for convenience so that their data may be compared to those of the normal subjects over 35 years of age shown in Table IV. The difference in D_{LCO} between these two groups, 37.4 in the younger and 30.5 in the older, is significant at the 2 per cent level, confirming work previously reported from this laboratory (8). However, the differences of 20 ml in V_c and 7.9 in D_M are not statistically significant in these small groups of subjects. More work will be required before the cause of the lowered D_L with age can be confidently attributed either to changes in V_c or D_M or both.

Validation of the theoretical considerations underlying this technique requires that the relationship between $1/D_L$ and $1/\theta$ should be linear.

TABLE III

*Determinations of pulmonary capillary blood volume and membrane diffusion components on seven normal subjects below the age of 35**

No.	Subject	Minute vol	$\dot{V}_{O_2}$	$\dot{V}_{O_2}/\dot{V}_{air}$	D_{LCO} (air)	D_{LCO} (O_2)	V_c	D_M	D_{LCO}/MC	V_c/MC	D_M/MC
		L	*L/min*				*ml*	*ml CO/min/mmHg*			
1	RED	25.3	1.21	4.79	38.8	21.4	163	58.2	10.0	42.0	15.0
2	CJV	31.7	1.27	4.01	41.4	25.2	200	56.8	10.3	49.5	14.1
3	FM	27.8	1.42	4.80	45.3	26.7	199	62.3	9.3	40.5	12.7
4	GW	31.9	1.11	3.38	39.7	22.2	152	60.2	9.1	34.7	13.7
5	DP	22.6	1.10	4.70	30.9	18.9	150	41.2	6.9	33.6	9.2
6	RF	25.9	1.01	3.41	29.0	15.9	104	45.0	8.5	30.2	13.1
7	JE	36.2	1.89	4.31	37.0	18.1	105	60.0	8.8	24.9	14.3
		22.6	1.01	3.38	29.0	15.9	104	41.2	6.9	24.9	9.2
	Mean†	28.8	1.29	4.20	37.4	21.2	153	54.8	9.0	36.5	13.2
		36.2	1.89	4.79	45.3	26.7	200	62.3	10.3	49.5	15.0
	SD	±4.35	±0.27	±0.57	±5.32	±3.12	±36.1	±7.7	±1.0	±7.5	±1.76

* All estimates done on exercise at 3 mph on a flat surface. Column headings are as in Table II.
† Figures above and below the mean value indicate the range of observations.

TABLE IV

*Determinations of pulmonary capillary blood and membrane diffusion components in the seven normal subjects above the age of 35**

No.	Subject	Minute vol	$\dot{V}_{O_2}$	$\dot{V}_{O_2}/\dot{V}_{air}$	D_{LCO} (air)	D_{LCO} (O_2)	V_c	D_M	D_{LCO}/MC	V_c/MC	D_M/MC
		L	*L/min*				*ml*	*ml CO/min/mmHg*			
8	DVB	28.4	1.21	4.26	35.2	16.7	96	66.5	9.2	25.3	17.4
9	LSB	23.7	1.04	4.39	22.1	11.9	68	40.2	7.4	22.9	13.7
10	REF	26.8	1.30	4.21	35.3	22.0	180	46.7	8.1	41.2	10.6
11	WF	27.6	1.03	3.51	32.6	17.4	114	51.7	6.9	24.4	11.1
12	JAP	25.8	1.18	4.11	30.2	19.8	176	46.0	7.9	38.2	10.0
13	REGP	29.5	1.17	3.83	22.5	12.5	83	33.1	9.1	33.4	13.4
14	JB	24.7	1.23	4.65	35.5	23.4	214	44.2	9.5	56.9	11.8
		23.7	1.03	3.51	22.1	11.9	68	33.1	7.4	22.9	10.0
	Mean†	26.6	1.17	4.14	30.5	17.6	133	46.9	8.3	34.6	12.6
		29.5	1.30	4.65	35.5	23.4	214	66.5	9.5	56.9	17.4
	SD	±1.89	±0.09	±0.28	±5.2	±4.18	±52	±9.6	±0.92	±11.2	±2.33

* Column headings are as in Table II.
† Figures above and below the mean value indicate the range of observations.

This relationship in Subjects DVB, CJV and RED is shown in Figure 1. The slope of the lines reflects the V_c component, and from the intercept, D_M is calculated. The general linearity of the points in any one individual is clear from this figure. The values of V_c and D_M calculated from these slopes appear in Table II. The data in Figure 1 have been used to predict the D_L at different values of $\bar{P}_{C_{O_2}}$ in the same three normal subjects during hypoxia, during voluntary hyperventilation, and during CO_2 breathing. In Table V are shown the results of measurements of D_L under these conditions in which little deviation could be demonstrated from the values of D_L predicted on the basis of Figure 1. This conclusion is discussed in detail later.

Patients. In Table VI are shown the physical characteristics of the first two groups of patients studied. Group 1 consisted of three patients who had had various parts of the lung removed, with

TABLE V

Effect of hyperventilation and hypoxia during exercise at 3 mph on a flat surface

Subject	Rate and grade	Experiment	Ventilation	Inspired oxygen% F_{IO_2}	Mean capillary O_2 $\bar{P}_{CO_2}$	Measured D_{LCO}	Predicted D_{LCO}*
			L/min			*ml CO/min/mm Hg*	
DVB	3 mph Flat	Hypoxia	44.3	10.7	22.0	41.8	41.2
		Hypoxia	40.5	12.4	30.9	43.5	40.6
		Hypoxia	35.6	12.8	34.9	39.4	39.7
		Hypoxia	32.3	15.0	39.9	32.6	39.3
		Hyperventilation	47.5†	20.8	96.3	36.0	34.8
		5.8% CO_2	48.3†	20.8	106.7	34.5	33.9
RED	3 mph Flat	Hypoxia	37.4	11.0	29.5	52.2	42.5
		Hypoxia	39.6	11.6	30.2	47.4	42.3
		Hyperventilation	51.3†	20.8	102.8	43.8	36.7
		5.8% CO_2	56.1†	20.8	95.5	46.4	37.7
CJV	3 mph Flat	Hypoxia	33.7	10.8	25.0	40.4	44.8
		5.8% CO_2	61.5†	20.8	113.2	45.0	40.7

* This column indicates the D_{LCO} predicted for the individual at the measured $\bar{P}_{C_{O_2}}$ (see Figure 1).
† See Table II for results at normal ventilations in these subjects.

TABLE VI

*Physical characteristics and resting pulmonary function tests in Groups 1 and 2 of the patients studied**

No.	Subject	Sex	Age	Ht	Wt	VC	FRC	ME%	MMFR	Diagnosis
				in.	*lbs*	*L*	*L*		*L/sec*	
	Group 1									
15	M FEN	F	50	65½	133	1.98	1.27	39	1.80	Pneumonect. L
16	J COT	M	41	69½	157½	2.12	1.87	47	1.50	Pneumonect. R
17	V RIC	F	27	68½	96½	1.41	2.13	33	0.52	Use of only rt. upper lobe
	Group 2									
18	A MAD	F	38	65	122					Thyrotoxicosis
19	G KOS	F	32	65½	120					Thyrotoxicosis
20	F BOU	M	35	64¼	132	2.80	2.72	64	1.31	Atr. sept. defect
21	I GAL	F	22	63	78	2.20	2.42	60	2.70	Atr. sept. defect
22	F ATK	F	30	63¼	100½	2.35	2.42	64	2.25	Pat. duct. art.
23	T NAD	M	20	69	155					Pat. duct. art.
24	A McC	M	37	71	152	1.65	3.32	34	1.10	Pat. duct. art.
25	H WAH	M	25	71½	149	2.96	2.78	67	1.30	Tric. atresia

* See Table I for details of column headings.

a reduction in FRC as a consequence. Group 2 consisted of a group of eight patients in all of whom there was reason to suppose that the pulmonary blood flow was abnormal; two of these patients had untreated thyrotoxicosis, two had atrial septal defects with high pulmonary blood flows, three had a patent ductus arteriosus with considerable increase in pulmonary flow in each case, and the last patient had tricuspid atresia with a reduction in cardiac output at rest as shown by cardiac catheterization. The results of determinations of V_c and D_M on these patients are shown in Table VII. In each case the exercise managed by the patient is shown in the second column and the minute volume and oxygen uptake are adjacent. The two patients who have had a pneumonectomy showed considerable variation in pulmonary capillary blood volume, though both values may be considered to be within half the approximate normal range. The membrane components are very constant and again are about half the predicted normal values for these subjects. The membrane component in relation to ventilated lung volume is slightly below the predicted values in these patients and the V_c in relation to lung volume appears to be normal in the first two patients and definitely reduced in the third (Patient 17). These findings are of some interest since

TABLE VII

*Results of determinations of pulmonary capillary blood volume and membrane diffusion components in patients in Groups 1 and 2**

Subject	Diagnosis	Rate†	Minute vol	$\dot{V}_{O_2}$	$\dot{V}_{O_2}/\dot{V}_{air}$	$D_{L_{CO}}$ (air)	$D_{L_{CO}}$ (O_2)	V_c	D_M	$D_{L_{CO}}/MC$	V_c/MC	D_M/MC
		mph	*L/min*	*L/min*				*ml*				
Group 1												
15 M FEN	Pneumonect. L.	1.5	19.90	0.80	4.00	12.3	6.5	47	14.9	6.3	24.0	7.6
16 J COT	Pneumonect. R.	2	21.60	1.05	4.85	16.7	11.1	103	19.3	7.3	44.0	8.4
17 V RIC	Use of only rt. upper lobe	1.5	15.67	0.53	3.38	11.5	6.0	38	18.3	4.7	15.3	7.5
Approx normal		3	21–31	1.0–1.9	3.5–4.8	22–45	12–27	68–200	33–66	6.9–10.5	24–56	9–17
Group 2												
18 A MAD	Thyrotoxicosis	1.5	18.65	0.73	3.91	17.3	11.6	111	21.2	6.9	44.3	8.5
19 G KOS	Thyrotoxicosis	2	20.50	0.88	4.29	19.5	11.0	79	27.6	7.1	28.9	10.1
20 F BOU	Atr. sept. defect	1.5	21.60	0.60	2.78	31.0	19.6	164	41.7	9.9	52.4	13.3
21 I GAL	Atr. sept. defect	2	22.65	0.55	2.43	30.2	18.6	150	41.7	10.4	51.5	14.4
22 F ATK	Pat. duct. art.	1.5	17.30	0.54	3.12	21.5	13.2	112	27.6	7.6	39.7	9.8
23 T NAD	Pat. duct. art.	2	24.85	0.97	3.90	22.7	14.1	113	30.0	6.8	34.0	9.0
24 A McC	Pat. duct. art.	1.75	35.60	1.09	3.06	21.9	13.7	109	29.0	5.5	36.3	7.5
25 H WAH	Tric. atresia	2	34.10	0.62	1.82	23.9	12.7	83	40.0	7.0	24.3	11.7

* See previous tables for details of column headings.
† All estimates done on exercise at rate shown on a flat surface.

TABLE VIII

*Physical data and resting pulmonary function tests in patients in Groups 3 and 4**

No.	Subject	Sex	Age	Ht	Wt	VC	FRC	ME%	MMFR	Diagnosis
				in.	*lbs*	*L*	*L*		*L/sec*	
	Group 3									
26	M LED	F	31	63	150					MS
27	H LAP	F	42	59½	79	1.95	2.34	40	1.27	MS
28	E MOF	F	34	57¾	102	2.50	2.37	67	3.50	MS
29	M HEA	F	46	62	104	2.16	2.51	59	2.20	MS
30	P MOL	M	19	70	155	3.94	2.83	50	5.70	MS + I
	Group 4									
31	E RYL	F	50	61	117½	1.45	1.58	48	1.40	DIF
32	E St.L	M	50	65	170	3.42	3.26	43	1.20	DIF
33	V AYB	F	48	64	140	1.87	1.41	59	2.40	DIF
34	S ARN	F	64	65	162	1.43	1.75	59	0.75	DIF
35	T LAV	F	46	63	113	1.70	2.38	52	2.50	BS
36	D BOU	F	15	67	116	2.75	3.07	63	2.70	BS

* See Table I for details of column headings. MS = mitral stenosis; MS + I = mitral stenosis + insufficiency; DIF = diffuse interstitial fibrosis; BS = Boeck sarcoid.

there is no evidence that the V_c of the remaining lung approximates that of the two lungs, although presumably it has a high pulmonary blood flow. The membrane components have been universally lowered by removal of one lung, as would be expected. Patients 18 to 24 may be presumed to have an increased pulmonary flow. The cardiac output is known to be much increased in untreated thyrotoxicosis during exercise (12), and a high resting pulmonary flow had been confirmed by cardiac catheterization in Patients 20 to 24. Patient 25 with tricuspid atresia was found at cardiac catheterization to have a cardiac index of half the predicted value. Consideration of the results in Group 2 as a whole shows that there is no evidence that an abnormally high or low pulmonary blood flow during exercise causes a change in V_c since all measurements of V_c in this group are within the predicted range. It is perhaps of interest that the membrane diffusing components (D_M/MC) were somewhat lower than expected in Patients 18 and 24, which might suggest that in some of these conditions there is an impairment of diffusion across the membrane resulting from the persistent high blood flow. In Table VIII are shown the physical characteristics and results of resting pulmonary function tests in patients in Group 3 and 4. Group 3 consists of four pa-

TABLE IX

*Results of determinations of pulmonary capillary blood volume and membrane diffusion component in patients in Groups 3 and 4**

Subject	Diagnosis	Rate†	Minute vol	$\dot{V}_{O_2}$	$\dot{V}_{O_2}/\dot{V}_{air}$	D_{LCO} (air)	$D\dot{V}_{CO}$ (O_2)	V_c	D_M	D_{LCO}/MC	V_c/MC	D_M/MC
		mph	*L/min*	*L/min*				*ml*				
Group 3												
26 M LED	MS	1.5	19.80	0.82	4.12	26.9	17.4	152	34.8	10.0	56.4	12.9
27 H LAP	MS	2	18.80	0.53	2.82	12.9	11.3	293	13.7	4.8	110.0	5.1
28 E MOF	MS	1.5	20.16	0.64	3.17	18.7	15.4	275	20.5	6.9	101.5	7.6
28 E MOF	MS post op.	1.5	13.63	0.60	4.36	21.3	11.9	84	31.3	8.1	31.1	11.5
29 M HEA	MS	1	26.60	0.53	2.00	14.3	7.7	50	23.0	4.8	16.9	7.7
30 P MOL	MS, MI	2	22.35	0.94	4.19	25.6	13.8	93	38.7	7.7	28.3	11.8
Approx normal		3	21–31	1.0–1.9	3.5–4.8	22–45	12–27	68–200	33–66	6.9–10.5	24–56	9–17
Group 4												
31 E RYL	CDIF	1.5	15.20	0.61	4.01	8.9	7.8	21	9.4	6.0	13.7	6.3
32 E St.L	CDIF	1.75	27.80	1.13	4.07	15.2	11.4	140	17.2	3.8	35.0	4.3
33 V AYB	CDIF	2	30.00	0.69	2.30	7.9	4.8	36	10.4	4.4	19.6	5.7
35 S ARN	CDIF	0.75	24.50	0.59	2.41	6.8	3.5	20	10.4	3.3	9.8	5.0
35 T LAV	BS	1.5	20.40	0.72	3.53	17.8	6.8	36	45.9	6.4	13.0	16.5
36 D BOU	BS	2	16.55	0.77	4.66	21.1	12.0	84	30.0	8.6	34.3	12.3

* See previous tables for details of column headings. MS = mitral stenosis; CDIF = chronic diffuse interstitial fibrosis; BS = Boeck sarcoid.
† All estimates done on exercise at rate shown on a flat surface, except those for Subject 36 who exercised on a surface with a 5 per cent slope.

tients with mitral stenosis and one with mitral stenosis with predominant mitral insufficiency. In Group 4 are four patients with chronic diffuse interstitial pulmonary fibrosis (in whom the diagnosis has been confirmed by lung biopsy in three), and two patients with sarcoid of the lung (in both of whom the diagnosis has been confirmed by lung biopsy). Table IX shows the results of determination of V_c and membrane components in these patients. In Patients 27 and 28, both of whom represented typical examples of tight mitral stenosis with considerable dyspnea and a reduced pulmonary compliance, the values of V_c are high, and those for the DM are low. Patient 28 was studied three weeks after a successful valvotomy, at which a very tight valve was found. On each occasion the level of exercise was the same, and there was an increase in $\dot{V}o_2/\dot{V}_{alv}$ after surgery from 3.17 to 4.36, in conformity with the observations of Cotes (13), and MacIntosh, Sinnott, Milne and Reid (14). There was a slight increase in over-all DL from 18.7 to 21.3, but this small change masks considerable alterations in V_c and DM. The V_c fell from 275 to 85 ml after surgery, and the membrane component rose from 20.5 to 31.3. Patient 29 had the murmur of mitral stenosis, but was judged to be so little incapacitated that surgery was not indicated. The pulmonary compliance in this patient was within normal limits (0.190 L per cm H_2O). It is of particular interest, therefore, that the V_c was normal, although there was a slight reduction in DM. In Patient 30, who had predominant mitral incompetence, the values for V_c and DM were within normal limits.

In Group 4 the changes are those one would expect to find in patients whose main difficulty is the transfer of oxygen across the pulmonary membrane. The DM is reduced in all patients in the pure pulmonary fibrosis group (Patients 31 to 34), and the extent of this reduction can be seen from comparison of DM in relation to ventilated lung volume in the last column. The values found in these four patients vary between 4.3 and 6.3 for this figure (DM/MC) whereas the normal range is from 9 to 17, approximately. Patients 35 and 36, with sarcoidosis, show considerable variation, the former having a normal DM but a low value of V_c, and the latter having a normal V_c and a nearly normal DM. These variations in this condition are to be expected, and the low V_c found in Patient 35 may reflect the fact that the main brunt of the lesion has been perivascular, as has been suggested by a number of authors (15, 16) for this condition. It is of interest, however, that the considerable difference in V_c between these two patients is not reflected by much difference in the over-all DL.

DISCUSSION

The data so far reported on the subdivision of the pulmonary diffusing capacity into its two components have been obtained by use of a single breath method (5–7). Apart from the objection that the holding of a deep breath may modify the quantities it is desired to measure, the single breath is difficult to manage during exercise. It is now generally recognized that the mean alveolar CO tension may be reliably measured during exercise in a steady state experiment, at least in subjects with normal gas distribution (8).

The present studies show that the components of pulmonary diffusion capacity may be reliably measured by this technique during steady state exercise, and the theoretical work of Forster and his colleagues has been confirmed by the demonstration under these conditions of a linear relationship between $1/D_L$ and $1/\theta$ (see Figure 1). It seems likely that the differences in the rate of gas diffusion between individuals at the same rate of exercise are mainly attributable to variations in the V_c. The DM, when expressed in relation to the volume of lung being ventilated, appears to be relatively constant in normal subjects at the same exercise level. The relative importance of these two factors is well illustrated by comparison of normal Subjects 2 and 6 in Table III. At the same exercise rate, the over-all DL was 12 units greater in Subject CJV; V_c in CJV was approximately twice that in Subject 6, yet the DM expressed in relation to lung volume was only 1 unit different between these two subjects. There was a large difference in V_c expressed in relation to the ventilated lung volume illustrating that the principal difference between these two subjects lay in this component rather than in the membrane component. Although these two subjects were the same age (see Table I), there was a 6 inch difference in height and a 60 pound difference in weight between them. There was only a 600 ml difference in vital capacity and a 700 ml difference in

FRC. The constancy of the results obtained in duplicate estimates of V_c and membrane components in the present study (Table II) may be compared to that reported by Lewis and associates (6) using the single breath technique. His data show less variation in V_c on repeat determinations, but the D_M showed more variation than that found in the present study. The reason for these differences is not altogether clear, but may reside in the errors inherent in the two techniques.

It may be pointed out, however, that provided the tidal volume is comparable during the two determinations of D_L, the value assumed for the respiratory dead space exerts no effect on the calculated V_c. Consequently, with a steady state technique of the type used, this subdivision of the D_L may be more reliably measured than the calculated D_M, which is affected by the value assumed for the dead space.

Ross, Frayser and Hickam (17), using the Filley method of calculating the mean alveolar CO tension, reported large increases in D_{LCO} with hyperventilation. These authors also suggested that much of the increase in D_{LCO} on exercise is to be attributed directly to the ventilation change. The data in Table V indicate that the observed values of D_{LCO} under conditions of hypoxia, voluntary hyperventilation, or CO_2 breathing, differ little from those that would be predicted for the individual concerned at the particular alveolar oxygen tension obtaining during the experiments. In only one subject (RED) did the observed values fall consistently above the predicted figures. No effect was demonstrable in the other two subjects. These results suggest that during exercise, neither hypoxia nor an increase in ventilation per se, causes any consistent or considerable alteration in the state of the pulmonary bed. The discrepancy between these observations and those of Ross and co-workers (17) and Turino, Brandfonbrener and Fishman (18), are not readily explained. Both authors reported striking increases in D_L during hyperventilation when the alveolar CO tension was computed from the measured arterial CO_2 tension. Such increases are not found if the end tidal CO is directly measured or if the mean alveolar CO is calculated, using a predicted value of respiratory dead space.

Forster (5) considered that anoxia might well change the pulmonary capillary bed, but if this were the case, the observed D_{LCO} in Table V under these conditions might be measurably greater than that predicted on the basis of the linear relationship shown in Figure 1. The precision of the data in Figure 1 in respect to these three normal subjects suggests that the line of observed relationship between $1/D_L$ and $1/\theta$ can be extrapolated confidently to cover low oxygen tensions, though θ values may not be reliable at capillary oxygen tensions below 150 mm Hg (2). The finding of little discrepancy between observed and predicted figures indicates that the effect of anoxia is probably slight, at least in short-term experiments on exercise, but more precise conclusions cannot be drawn from the data at this stage.

The study of the 22 patients in the present series leads to the following general conclusions.

1. When portions of the lungs are removed, D_M is proportionately reduced. The V_c of the remaining lung seems variable. The general result is that the over-all D_L is about that predicted for one lung, as was found in previous studies of McIlroy and Bates (19) and Linderholm (20). There does not appear to be any considerable increase in the V_c of the remaining lung in relation to its gas volume, although presumably the pulmonary blood flow through the lung is substantially increased.

2. In long-standing conditions in which the pulmonary blood flow is greatly increased, there is no concomitant increase in V_c. This conclusion is somewhat at variance with that of Auchincloss, Gilbert and Eich (21), who used the single breath D_L technique at rest to study patients with congenital and acquired heart disease. These authors found an increased D_L at rest in some patients in the congenital heart disease group, but they did not report any exercise results. Their findings are therefore not necessarily at variance with the present conclusion that the exercise D_M and V_c are not increased in these patients compared with those of normal subjects. The possibility exists that the taking and holding of a single breath by patients with large intracardiac shunts may significantly alter the state of the pulmonary bed. The finding of a lowered D_M in Patients 23 and 24 may well reflect irreversible changes secondary to the pulmonary hypertension, since the two patients with primary pulmonary hypertension studied by McNeill and associates (7) showed a de-

crease both in D_M and in V_c. The pulmonary diffusing capacity in thyrotoxicosis has been noted previously to be normal or lower than normal, even though the pulmonary blood flow is considerably elevated.[1] The two patients with untreated thyrotoxicosis included in the present study illustrate this finding. The predicted $D_{L_{CO}}$ for these two patients at the exercise level they maintained is 27 and 31 ml CO per minute per mm Hg, respectively (8), whereas the measured figures were 17.3 and 19.5. The D_M was reduced in the first of these patients and was clearly limiting the rate of gas diffusion considerably. In the second patient it was just below the approximate normal level. In neither patient was there any increase in V_c. Taken together, the findings in Group 2 in Table VII indicate that the components of pulmonary diffusion are independent of the absolute level of pulmonary blood flow. This supports the animal experimentation reported by Rosenberg and Forster in which the authors arrived at the same conclusion (22).

3. The finding of high values for V_c in mitral stenosis supports the conclusion of McNeill and associates (7). The additional evidence presented above suggests that this may be an important measurement in this condition, since the over-all D_L may alter little after a successful valvotomy and so conceal the fact that the V_c and D_M components have changed considerably in different directions. The variable results noted by others (14, 23, 24) in the over-all D_L in mitral stenosis, may well be explained by the present observation that further subdivision of the D_L is required before the data become meaningful in terms of changes in the pulmonary capillary bed. Further, the present finding of normal values in a patient with little disability, who was known to have a normal pulmonary compliance, certainly suggests that alterations in V_c and membrane diffusion components represent a basic change in the physiopathology of this disease. The demonstration of a marked change in these values only three weeks after a valvotomy (Patient 28, Table IX), is also of considerable interest, since it has proved difficult to correlate the considerable subjective improvement which many such patients have with any measurable physiological variable. It may be that the measured V_c during exercise will prove to be a sensitive indicator of change occurring in the pulmonary bed as a result of valvotomy.

4. In pulmonary fibrosis the measurement of D_M probably represents the most sensitive criterion of normality available. Its expression in terms of the ventilated lung (D_M/MC) increases its sensitivity considerably. This is well illustrated by Patient 32 in Table IX whose pulmonary infiltration was known to have been present for less than three months. The D_M/MC ratio was 4.3 against the predicted value of at least 9.0. The lung biopsy in this patient showed that the alveolar walls were uniformly thickened but only by about one extra layer of cells. Pathologically the section was regarded as representing an extremely early example of Hamman-Rich syndrome. Systematic study of D_M changes in this and other types of pulmonary fibrosis would undoubtedly provide a sensitive indicator of alterations in the pulmonary membrane. In certain diseases such as sarcoidosis, it may be found that the main area of involvement in the lungs can be localized by a study of the relative changes in V_c and D_M. The finding in the present study of an unexpectedly low V_c in a patient with sarcoidosis, as well as in two of the patients with a diffuse interstitial fibrosis, suggests that the involvement of the lung may be quite different in different patterns of these diseases.

[1] Bates, D. V. Unpublished observations.

SUMMARY

1. The components of the pulmonary diffusing capacity have been studied in 14 normal individuals, and in a group of 22 patients with varying clinical conditions. All the determinations of pulmonary capillary blood volume and membrane diffusing capacity were made during exercise.

2. The results reported show that this determination is sufficiently stable and reliable to permit comparisons to be made between normal subjects and patients.

3. The data suggest that in normal subjects the pulmonary diffusing capacity increases with exercise mainly because the pulmonary capillary blood volume rises. Individual differences in diffusing capacity also seem mainly attributable to variations in this component. Hyperventilation and hypoxia

could not be shown to exert much influence on the pulmonary diffusing capacity.

4. Removal of one lung appears to result in a halving of the membrane component.

5. High flow conditions in the lesser circulation are not associated with any increase in pulmonary capillary blood volume. In one patient with a reduced pulmonary flow the pulmonary capillary blood volume was normal.

6. Mitral stenosis may lead to a considerable increase in pulmonary capillary blood volume, and a concomitant reduction in membrane component. In one patient studied before and after a successful valvotomy, the pulmonary capillary blood volume at equivalent exercise had dropped from 275 ml before operation to 85 ml postoperatively and the membrane component rose from 20 to 31 ml CO per minute per mm Hg. This reciprocal change in these two variables resulted in a very similar over-all diffusing capacity before and after operation.

7. In a group of patients with pulmonary fibrosis the main defect was found to be in the membrane diffusion component, the pulmonary capillary blood volume being either normal or reduced.

8. It is concluded that this technique based on the theoretical work of Forster (5) is capable of yielding valuable information in a wide variety of clinical conditions.

ACKNOWLEDGMENTS

We wish to thank members of the Medical and Surgical Staff of the Royal Victoria Hospital who referred patients to us for study. Mr. L. S. Bartlett, Mr. L. D. Pengelly and Mr. L. Nowaczek provided valuable technical assistance during this work.

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ARTERIAL BLOOD FLOW PATTERNS IN HUMAN SUBJECTS AND THEIR EFFECT ON INDICATOR DILUTION CURVES FROM VARIOUS ARTERIAL SITES *

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Indicator dilution curves obtained from various systemic arterial sampling sites are commonly used for analysis of blood flow, cardiac shunts and valvular regurgitation, and for study of segments of the blood volume. It is generally assumed that the contour of such curves is largely determined by the passage and dilution of the indicator in the central circulation and that changes occurring during arterial passage are minor and predictable on the basis of the expected velocity of arterial blood flow and the distances of the commonly used sampling sites from the aortic root. Although it is accepted that quantification of blood flow is unaffected by changes in curve contour so long as the first circulation of indicator can be identified, the estimation of other parameters available from curve analysis may be significantly influenced by the validity of the above assumption. Beard, Wood and Clagett (1) and Fox, Sutterer and Wood (2) have demonstrated considerable differences in contour as well as appearance time of curves obtained from the femoral (FA), radial (RA) and dorsalis pedis arteries. The first study showed delay in the FA components in coarctation of the aorta as compared with the RA curve, whereas the latter study noted delay and skewing of the RA or pedal curve as compared with the FA curve. The FA curves were similar in contour to the abdominal aortic curves in normal subjects. During preliminary studies of an investigation which attempted to quantitate valvular regurgitation by comparison of indicator dilution curves recorded simultaneously from the pulmonary artery (PA) and a systemic artery, we noted that in normal subjects only FA curves consistently resembled the PA curve while those recorded from the brachial artery (BA), or RA frequently showed unpredictable deviations in curve parameters.

Although these studies clearly indicate that the sampling site as well as the sampling system (3–6) play an important role in the parameters of dilution curves, it is not clear whether the effects of arterial passage are a predictable characteristic in each arterial system and are constant from subject to subject and from moment to moment, or whether capricious effects occur which are related to spontaneous or reflex local disturbances, possibly produced by the sampling technique.

The present study was undertaken in an attempt to define the extent of variation in curves as obtained from commonly employed arterial sites and to examine the possible mechanism for distortion or skewing of the curves. The results of human studies led to the parallel investigation in a hydraulic analog system having three characteristics considered most important in determining the presence and extent of curve alteration: pulsatile flow, a branched elastic system, and variable efflux resistances. Although the effects on a dilution curve of constant flow through rigid tubing are well known (3–6), little evidence exists as to the pulsatile flow through a branched elastic system encountered in man.

METHODS

Human studies. Studies were carried out in 12 human subjects in the resting state during the course of venous cardiac catheterization. Simultaneous FA and BA curves were obtained following right heart or venous injection of T-1824 or indocyanine green in 4 normal subjects. In 3 others, simultaneous RA and FA curves were obtained (Table I, Subjects 1 through 7). Three patients with coarctation of the aorta were examined: one with minimal narrowing showing only alteration of the central pressure slope (Patient 8), and two with evidence of severe constriction of the aorta and well developed collateral circulation (Patients 9 and 10). An-

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TABLE I

*Parameters of simultaneous dilution curves from two arterial sites**

Subject no.	Age, Sex	Diagnosis	Sampling sites†	Δt_a‡	Δt_p‡	$\Delta(t_d-t_a)$‡
1	21, M	Normal	FA/BA	+2.3	+3.5	+5.5
2	18, M	Normal	FA/BA	+2.0	+4.0	+6.0
3	30, M	Normal	FA/BA	+1.0	+1.5	+1.0
4	36, M	Normal	FA/BA	0.0	0.0	+1.0
5	29, M	Normal	FA/RA	+3.5	+4.7	+6.0
6	23, M	Normal	FA/RA	+6.0	+8.5	+10.5
7	29, M	Repaired atrial septal defect	FA/RA	+3.0	+3.0	0.0
8	24, M	Funct. coarctation aorta	FA/BA	+1.0	+1.5	+1.0
9	38, M	Severe	FA/BA	−1.0	−2.0	−5.0
10	19, M	Moderate	FA/BA	−1.0	−1.5	−1.0
11	66, M	Abd. aortic	FA/BA	−8.0	−9.0	−2.0
12	23, F	Bradycardia	FA/BA	0.0	0.0	0.0

* Records were obtained by using either two direct recording oximetergalvanometer systems or two densitometer-galvanometer systems (see Methods).

† FA, BA and RA refer to curves obtained from the femoral artery, brachial artery and radial artery. In Subject 9, the left brachial artery was used.

‡ t_a, t_p and t_d-t_a refer to appearance time, peak concentration time, and passage time, respectively, following the nomenclature proposed by others (2). Δt_a, Δt_p and $\Delta(t_d-t_a)$ refer to the relationship between values of the two simultaneously recorded curves. Positive sign indicates that FA curve measurements were the smaller.

other subject (Patient 11) had a large abdominal aortic aneurysm and Patient 12 was included because of a bradycardia of 36 beats per minute. FA and BA curves were obtained in Patients 8 through 12.

Arterial punctures were performed following liberal infiltration of the puncture site with 2 per cent procaine; 20 gauge needles were used for the radial and 18 gauge or 20 gauge needles for the brachial and femoral punctures. None of the patients complained of discomfort from the indwelling needle. The device for simultaneous sampling from two sites is described elsewhere. A sampling rate of 0.3 to 0.6 ml per second was used in all studies. Cuvet oximeters [1] or cuvet densitometers [2] used were attached directly to the arterial needles, and the delay times due to the dead space of cuvet tubing (0.5 ml in the oximeter and 0.25 ml in the densitometer) were equal and less than one second. The curves were recorded by a kymographic camera [3] at a paper speed of 5 mm per second. The downslopes were extrapolated to points of equal concentration.

Paired curves were analyzed for the following parameters: appearance time, t_a, and peak concentration time, t_p. The "disappearance time" was estimated by extrapolating the curve with the most rapidly decaying concentration to 0.05 of the concentration at the beginning of apparent exponential decrease in concentration. The other curve is then extrapolated to an equal concentration. This is taken to be the time when the two sampling sites reflect a common event occurring upstream in the aorta.

In addition to the experiments described above, arterial blood flow patterns were studied in 8 normal subjects and in 3 individuals with valvular heart disease who were free from obvious arterial disease. This was accomplished by observing with cinefluorography [4] the distribution and flow of 50 per cent diatrizoate sodium (Hypaque) introduced into an artery. Following percutaneous puncture with an 18 gauge needle, a specially modified 20 gauge needle, 10 cm long with the tip occluded and fenestrations placed radially over the distal centimeter of length was passed through the no. 18 needle so that the multiple openings lay within the lumen of the vessel studied. After free flow was ascertained, cinefluorographic recordings were made of the streaming pathways of slowly injected, 1 ml per second, and rapidly injected, 2 to 4 ml per second, 50 per cent Hypaque. In 4 subjects injections were made into the brachial artery. The femoral artery was used in 4 normals and in 3 patients with mild aortic valvular incompetence. In 3 of the normal subjects in whom FA injection was made, records were obtained before and after occlusion of arterial blood flow just above the knee by means of a pneumatic cuff inflated to suprasystolic pressure.

Model studies. The hydraulic analog consisted of a simple branched system of rubber tubing with a small glass "Y" tube at the bifurcation. Water was pumped through the system by means of a Sigma Motor finger pump, which delivered pulsatile flow with a stroke volume about 10 ml at a rate of 80 per minute. The volume of the undistended system measured, under various con-

[1] Wood cuvet oximeter, Waters Corp., Rochester, Minn.

[2] XC-100A densitometer, Waters Corp., Rochester, Minn.

[3] Waters kymographic camera, model G-12, Waters Corp., Rochester, Minn.

[4] Cinefluorex, Westinghouse Corp.

ditions, from 16 to 20 ml. The distal ends of the two branches of tubing could be controlled by screw clamps to provide variable outflow resistances. Indicator was injected proximal to the pump. The latter provided adequate mixing. Dilution curves were obtained through short no. 20 needles connected to direct recording cuvet oximeters. Sampling was accomplished by the paired sampling syringes mentioned previously (3). The rate of sampling at all times was less than 5 per cent of the flow through the model. The model was intended to closely simulate the relative capacity of the arterial tree and the flow rate through it. The following paragraph describes the manner in which local alterations in flow were simulated.

The following experimental conditions were set up to test their influence on the contour of a dilution curve: *a*) sampling from two branches having equal flow, but equal or unequal volume; *b*) sampling from two branches having unequal flow, accomplished by changing outflow resistance of one branch only; *c*) sampling from proximal and distal segments of one branch having increased outflow resistance; *d*) sampling from a nonexpansile system made so by lowering outflow resistance in both branches; *e*) sampling from both branches with elevated, but slightly unequal, outflow resistances.

The failure of the galvanometer beam to return to the baseline after curve inscription deserves mention. It was noted that the polyethylene tubing, leading water and T-1824 dye through the recording cuvet, would remain faintly tinted with blue color long after the effluent was

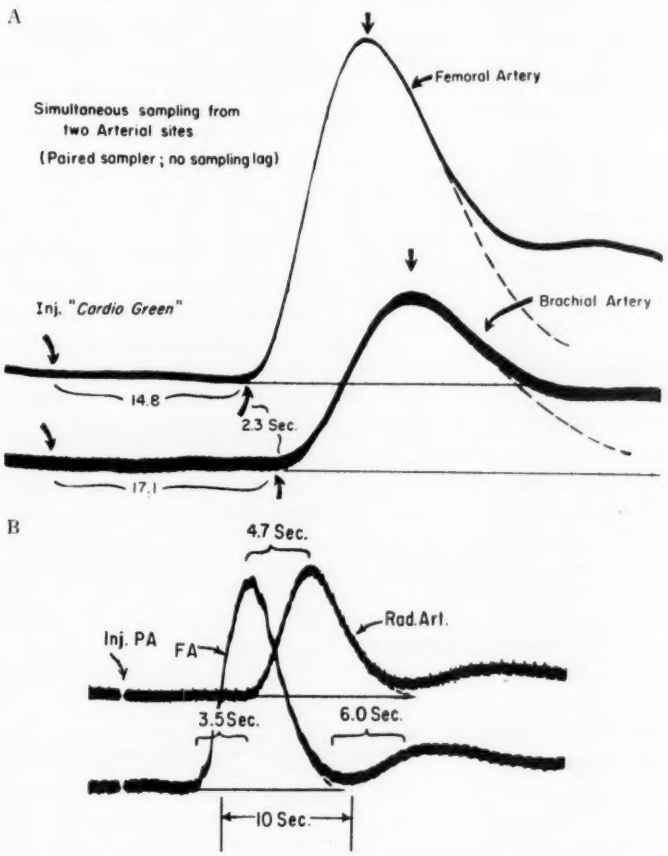


FIG. 1. A. A CHARACTERISTIC SET OF CURVES FROM A NORMAL MALE (SUBJECT 1, TABLE I) SHOWING FAIRLY EXTREME VARIATION BETWEEN BA AND FA CURVES. Curves are extrapolated to equal concentrations. B. SIMULTANEOUS CURVES FROM THE FA AND RA FOLLOWING PA INJECTION. Recordings were made using cuvet densitometers sensitive to indocyanine green. The variation of the RA curve from the FA curve is qualitatively the same as the variation of the BA curve from the FA curve in 1A.

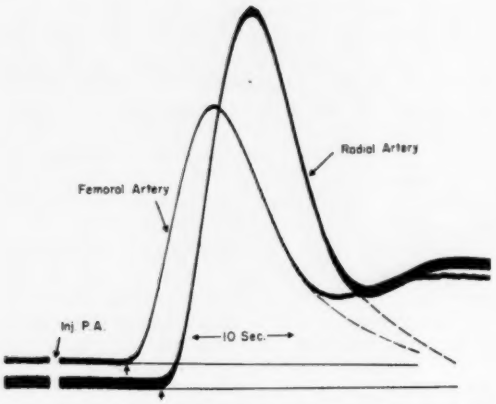


FIG. 2. CURVES FROM FA AND RA IN A 29 YEAR OLD MALE (SUBJECT 5, TABLE I) FOLLOWING SUCCESSFUL REPAIR OF AN ATRIAL SEPTAL DEFECT. Note the delay of all parameters of the RA curve without skewing.

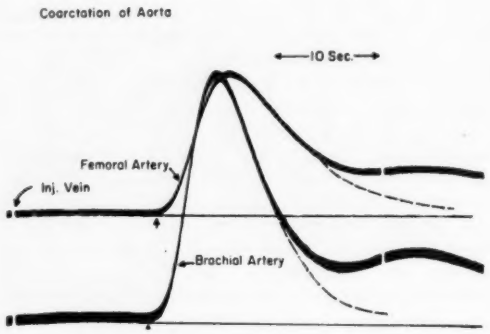


FIG. 3. THE EFFECTS OF SEVERE MECHANICAL OBSTRUCTION OF AORTA DISTAL TO THE LEFT SUBCLAVIAN ARTERY (SAMPLING FROM THE LEFT BRACHIAL ARTERY). The delay and skewing of the FA curve as compared to the BA curves is probably the result of increased U/F ratio distal to the coarctation and to the presence of numerous collaterals with variable transit times.

colorless. We conclude that loose binding between the dye and plastic occurred with a significant effect only at low concentration. We, therefore, doubt the accuracy of the downslope at lower concentrations and have used the segment of the curve shortly after the peak for comparative purposes.

RESULTS

1. Comparison of human curves obtained simultaneously from two arteries. As demonstrated in Figures 1, 2 and 3, considerable variability is encountered when two curves are recorded simultaneously from various arterial sites following single venous or right heart injection.

The measured parameters of paired curves are listed in Table I. There is frequently not only delayed appearance, t_a, of dye at the BA or RA when compared with the FA, but in addition the curve is skewed by further delay in the peak concentration time, t_p, and prolongation of the passage time, t_d-t_a (Subjects 1, 2, 5 and 6). These findings agree in general with those of Fox, Sutterer and Wood (*2*), but there is not always significant alteration of the BA curves (Subjects 3, 4, 8 and 12) and the RA curve may be delayed without other alteration (Subject 7). Examination of the pair of curves in Figure 1A shows that the delay in t_a is about 2.3 seconds, the delay in t_p 3.5 seconds and the increase in t_d-t_a is even greater, 5 seconds. Thus, the first appearing dye is delayed at the BA site, but not so much as is the dye par-

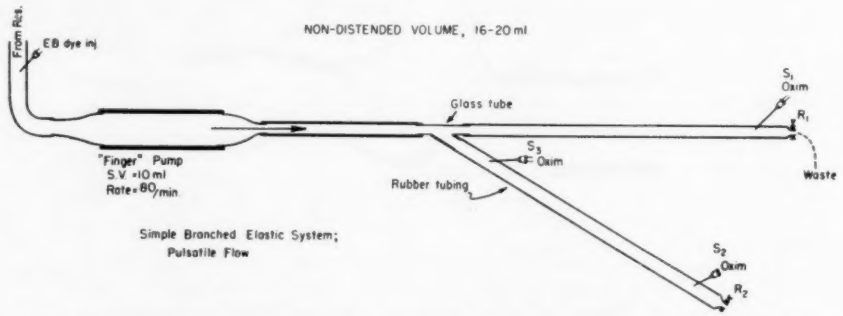


FIG. 4. THE OUTLINE OF THE MODEL OF A SINGLE BRANCHED SYSTEM WITH EXPANSILE AND PULSATILE FLOW. Ratio of "diastolic" volume to stroke volume and pump rate are close to these values expected in the human arterial tree. R and R_2 are variable resistances; S_1, S_2 and S_3 are sampling sites.

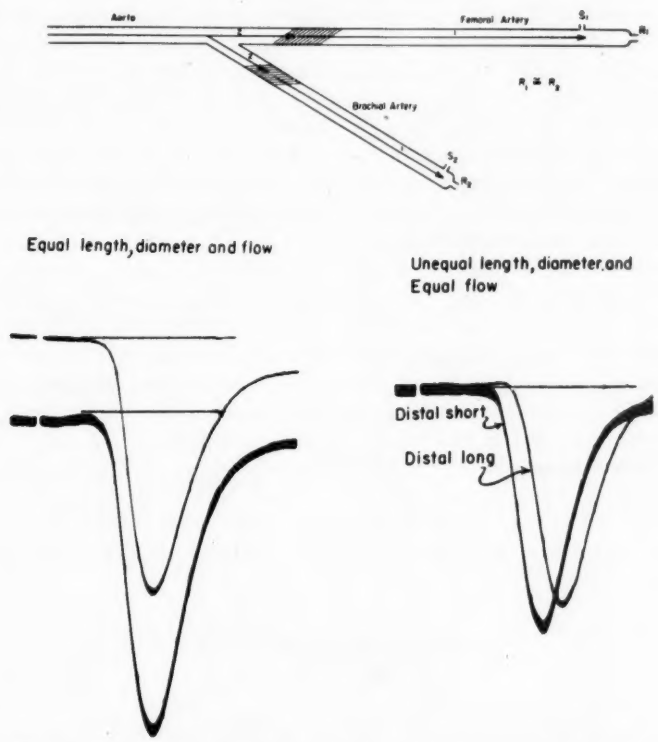


FIG. 5. TWO SUCCESSIVE STROKE VOLUMES FROM A DILUTION CURVE, EACH STROKE A RATHER HOMOGENEOUS "STEP FUNCTION" OF CONCENTRATION. The shaded areas indicate that the mixing length is short compared to the distance over which concentration tends to be constant. This concept would explain the finding of nearly constant contour of curves from various portions of a system where U/F ratios were equal in the two branches. Below, representative curves from such a system are shown.

ticle with average transit time, $\bar{t}$, and even less than the particles near t_d. Figure 1B, recorded with more rapidly responding cuvet densitometers from the FA and RA, shows similar findings. The cases included in Table I represent all possible combinations of FA and BA and RA relationships: *a*) the common occurrence of delayed appearance and skewness of the BA with respect to the FA (Subject 1 and 2); *b*) no significant difference between FA and BA curves (Subjects 3, 4, 8 and 12); *c*) delay in onset, t_a, of the RA curves with or without variation of contour (Subjects 5, 6 and 7); *d*) reversal of usual findings in severe coarctation (Subjects 9 and 10) and in abdominal aortic aneurysm (Subject 11).

2. Cinefluorex studies. In all FA studies including those of three subjects with mild aortic regurgitation, Hypaque, injected slowly at 1 ml per second, was carried distally without visible diastolic retrograde movement. During repeated studies, or if Hypaque was injected at a faster rate for 8 to 10 seconds, a variable degree of retrograde movement, never more than 3 to 5 cm, was apparent in diastole. There was always rapid clearance of injectate from the site.

In the three FA studies with cuff occlusion of the arterial flow just above the knee, striking retrograde flow of Hypaque was seen as far proximal as the aortic bifurcation. Subsequent injections with the cuff released showed the relationships seen in the control period.

Brachial artery injections revealed a more variable pattern. In one patient there was no visible retrograde movement. In two remaining normal

subjects, there was a variable diastolic retrograde movement of injectate with slow clearing of indicator after cessation of injection. In one normal subject, a brief rapid injection of a small volume resulted in retrograde flow so striking that the subclavian artery was opacified and, although radial pulse was palpable, washout time was prolonged to 18 seconds after cessation of injection.

3. Model studies. The outcome of Studies 1 and 2 above suggested the simple branched elastic model (Figure 4) with pulsatile flow and the variations of the volume-flow relations described above. When equal flow occurs through equal volumes of the pulsatile system, the curves obtained from each branch are identical in all parameters. When the volumes to be traversed are unequal but the flow rates are identical, the similarity of the two curves is maintained, but of course the distal curve has delayed inscription (Figure 5). Thus, differences in volume contained in the two branches of the system do not significantly change the contour of the curves. However, a disproportionate decrease in flow of one branch ("vasoconstriction"), with volume, elasticity and stroke volume kept constant, shows that, in addition to the expected delay in the appearance time, the peak concentration time and the extrapolated passage time are further significantly delayed (Figure 6). In fact, when compared to the delay in t_a, t_p and t_d show even greater "smearing," i.e., they demonstrate progressive delay when compared to the same measurement of the branch of the model that had normal outflow resistance. This same relative difference between t_a, t_p and t_d was noted in many human subjects when simultaneous sampling from radial or brachial artery was compared with the femoral artery.

With high outflow resistance of one branch of the model, similar differences appear when curves are recorded simultaneously from the proximal

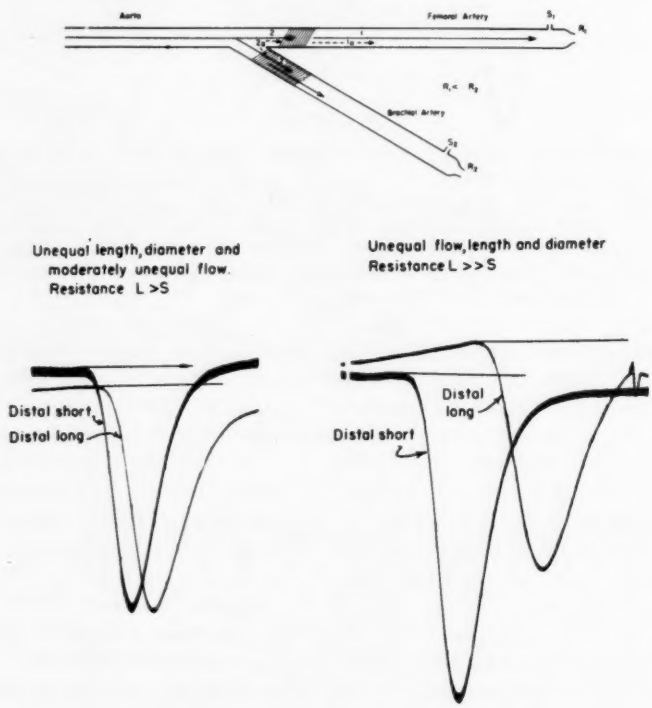
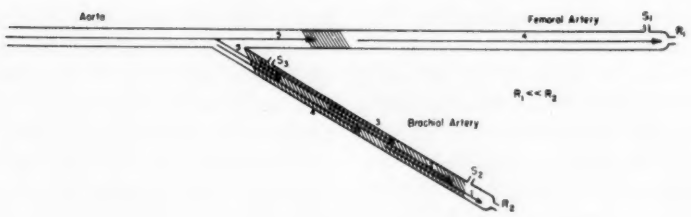


FIG. 6. SCHEMATIC REPRESENTATION OF INCREASED U/F IN A SIDE BRANCH ACCOMPLISHED BY INCREASING RESISTANCE R_2. Now the mixing length is an appreciable fraction of the total segment occupied by the aliquot of stroke volume. Below are seen the effects of two conditions of unequal flow.



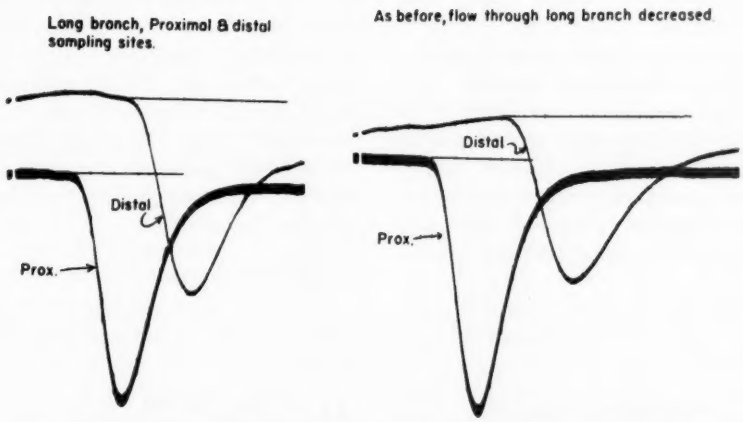


FIG. 7. THE DEPICTION OF MIXTURE OF SEVERAL ALIQUOTS OF SUCCESSIVE STROKES IN THE HIGH U/F SIDE BRANCH. Thus the fourth stroke concentration may be recorded from the FA (or S_3) while the first stroke concentration reaches the site S_2. Strokes 1 through 5 intermingle, strokes 1 and 2 mix "ahead" with undyed blood and will be recorded in advance of the centroid of the curve. After t_p, this process is reversed with volumes of lower concentration entering the segment and mixing with higher concentrations present; in consequence delayed clearance occurs. Note the similarity of curves from S_3, the proximal site on the side branch, and S_1 of Figure 6.

and the distal end of the same branch (Figure 7). The proximal curve is similar to a record obtained from a branch with normal outflow resistance; the distal curve is similar to that from the constricted branch of Figure 6, so that the difference in curve parameters sampled from these two sites of the same "vasoconstricted" branch is essentially the same as the difference obtained from one normal and one constricted branch. This is more marked when the resistance in the sampled tube is further increased.

Finally, conditions of flow in a non-expansile system were simulated by causing such low outflow resistance that discontinuous (systolic) rather than continuous but pulsatile flow occurred. This solely systolic and presumably laminar flow did not cause distention of the rubber tubing and no appreciable elastic effect remained (Figure 8 left). Under these circumstances no distortion of contour was found. Likewise, when extreme, although unequal increase in resistance was applied to the two branches, there again was no distortion of proximal and distal curves obtained from one branch (Figure 8 right).

DISCUSSION

From the results obtained in the human studies, it is apparent that, in the absence of structural abnormality of the aorta, the FA curve invariably has values of t_a, t_p and t_d-t_a equal to or less than those of a simultaneous curve from another arterial site. Published curves of Fox and associates (2) indicate that the FA curve does not differ significantly from the curve obtained from the

abdominal aorta except for the expected brief and equal delay of all parameters. Comparison of curves obtained from the PA and FA following venous injection in normal subjects shows only a small, nearly constant alteration in contour (7). These observations support the conclusion that, when dealing with a normal arterial tree, the FA curve may be considered a close representative of its antecedent as it emerged from the left ventricle. The reason this site is unique among those commonly utilized may be the nearly pure forward flow patterns generally found with FA radiographic studies, except when mechanical obstruction is induced distal to the site studied. As a corollary, of all commonly used sites in man, the FA curve can be recorded with a minimum of delay and is not likely to imply a "Stewart" volume greater than the anatomical volume existing between aortic valve and sampling site. This conclusion is not warranted if gross aortic disease exists.

Curves from the BA may or may not differ from the FA curve in an apparently unpredictable manner. This correlates with variability of flow patterns seen in BA injection studies. Although the number of paired curves from the FA and RA is small, the latter curves show the most striking alterations and this is in keeping with the finding of slow clearance of radiopaque substance distal to the BA injection site even though severe alterations of flow patterns were not seen at that site.

The manner in which curves may be altered by arterial passage would seem to depend on local variation of flow and volume relations. The observed facts implicate two mechanisms, both of which are the consequence of local vasoconstrictive action: local regurgitation (return flow) because systolic uptake exceeds the runoff capacity of the segmental vascular bed, and a smaller systolic uptake over a prolonged time period because of a decrease in the compliance of the segment of arterial bed involved.

A suggested skewing mechanism

As stated, any explanation advanced for the mechanisms by which curves may be altered dur-

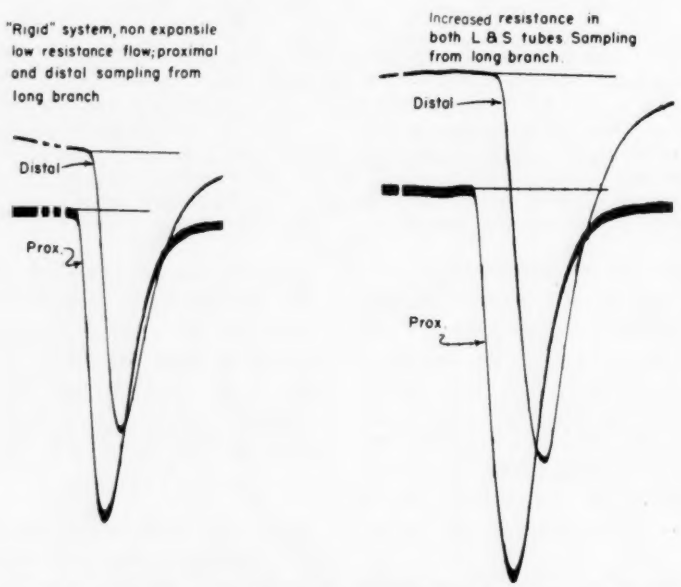

"Rigid" system, non expansile low resistance flow; proximal and distal sampling from long branch
Distal
Prox
Increased resistance in both L & S tubes Sampling from long branch
Distal
Prox

FIG. 8. (LEFT) THE EFFECTS OF PULSATILE BUT NONEXPANSILE FLOW. There is no discernible effect due to laminar flow in the side branch. (RIGHT) EXTREMELY HIGH RESISTANCE IN BOTH BRANCHES; PULSATILE, EXPANSILE FLOW. Again only delay is seen in all portions of the curve from the distal site without skewing. This is similar to the curve from human subject, Figure 2.

ing passage through the arterial tree must include considerations of a pulsatile elastic system. It must also embrace the premise that the normal arterial tree receives the dilution curve in successive stroke volumes of more or less homogeneous concentration which occupy segments along the tree which are long as compared to the distance over which longitudinal mixing can occur during the time of arterial passage. To be acceptable, longer mixing pathways, shorter length of constant concentration, longer time of passage, or a combination of these must be incorporated in the explanation. The following paragraphs and Figures 5 through 8 attempt this.

First, consider the various branches of the arterial tree as having a diastolic runoff equal to the net systolic uptake. If this ideal condition prevails, then all the blood entering a given branched segment in systole will eventually leave the appropriate drainage bed at the other end (Figure 5). To the extent that the ratio of systolic uptake or "capacitance" over diastolic runoff or flow (U/F) of a branched segment differs from the ideal situation, more or less blood will leave the drainage bed in diastole than the net amount which entered during systole. If the U/F ratio is greater than average, then some of the systolic uptake which has penetrated the segment a certain distance will not be able to proceed and will flow or regurgitate into other segments with smaller U/F ratios. This circumstance, illustrated in Figures 6 and 7, would allow a degree of regurgitation in any segment in which the U/F ratio exceeded the average. The effects of this regurgitation on the remaining low U/F vessels would not necessarily be discernible, since the concentration of indicator is identical in all of the systolic ejectate and subsequent diastolic runoff.

The effects of this localized regurgitation can be examined further. Let us consider the course of events which follows the end of systole. (Assuming the pressure is nearly the same at all points in this small segment, one may consider diastole to begin at nearly the same time in all parts.) Consider first the fate of the blood immediately adjacent to the arterioles; this will flow continuously forward during diastole and in this it is similar to all the juxta-arteriolar blood. However, the blood and indicator, which at the end of systole are found at the entrance to this segment, reverse flow and enter another drainage area. The fate of blood at various intermediate points will generally fall between these extremes and theoretically there is a point in such a segment where the two opposite effects balance and no net flow occurs during diastole. These reasonable conclusions thus far have not caused an alteration of the time concentration curve as it traverses this aberrant segment since, at any given instant, the concentration is the same in both average and aberrant segments. A second abnormal condition is needed for dispersion to occur and to result in different concentrations in the two segments at a given time. This may be related to the manner in which systolic "penetration" occurs. In any segment a certain end-diastolic volume exists. The systolic uptake is smaller than the end-systolic volume, and to the extent that the latter exceeds the former, more than one stroke will be required until the indicator substances have completely traversed a segment. If diastolic flow is normally related to net systolic uptake, i.e., equal ($U/F = 1$), then the average traversal time may be considered equal to the volume of the segment divided by the arteriolar runoff per second. It follows that with diminished runoff and constant volume, this time would increase inversely as the runoff decreased. Note that this will allow a delayed t_a as seen in human and model curves, but not necessarily an alteration of the contour. This situation is encountered in Table I, Subject 7, and in Figures 2 and 8. A further essential is the distribution of small aliquots of each stroke volume (systolic ejection) over an additional volume (and between one another). Since it has been stipulated that the stability of the curve as normally seen is due to a short longitudinal mixing distance and the relatively much greater length of homogeneous concentration, it is possible to increase the dispersion and alter the curve either by decreasing the length of homogeneous concentration, by increasing the length of longitudinal exchange, or by allowing longer time for longitudinal mixing. When small aliquots of aortic ejectate enter a segment with increased ratio of U/F so that several successive short subsegments have different concentrations, and if conditions within the segment allow a significant degree of longitudinal mixing, we finally have all three conditions necessary for definite alteration of the input curve. The cir-

cumstances encountered in an "average" U/F segment are shown schematically in Figure 5. It will be noted that there are long distances of homogeneous concentration and short distances over which mixing can occur. This situation is contrasted with a high ratio U/F segment in which the distance between blood with two concentration levels may be of the same order as the distance of mixing (Figures 6 and 7).

Correlation of the theoretical mechanism outlined and the observed abnormalities follows. As successive small volumes of increasing concentration enter the segment of high U/F and move more slowly, as in Figures 6 and 7, a delayed $\bar{t}$ is to be expected. However, the mixing effect will cause an apparent "advance" of the appearance time t_a, since it is delayed less than $\bar{t}$; $\bar{t}$ is not affected by the mixing process which is bidirectional and of equal magnitude. Since t_a is delayed less than $\bar{t}$, the first part of the curve is longer than the simultaneous curve recorded from the undisturbed segment. After peak concentration is reached, and decreasing concentration occurs with successive strokes, the mixing of aliquots of several strokes now slows the clearance, i.e., t_d-t_p is longer in the segment with a high U/F. The human studies, Figure 1A and B, and model studies, Figures 6 and 7, are equivalent examples of this.

Of interest is the effect of mechanical obstruction on the contour of the dilution curve. Subject 8 (Table I), a patient with mild aortic coarctation, exhibited little functional evidence of obstruction (8) and showed the usual relations between FA and BA artery curves. Subject 9, with severe but incomplete obstruction, exhibited delay and skewing of the FA curve as compared to the BA curve, the reverse of the usual finding. This alteration of the FA curve is probably primarily caused by the increased U/F ratio distal to the obstruction rather than by the presence of collateral pathways of variable transit times. This is suggested from the evidence that the alteration of the FA curve is similar to that seen in BA when increased U/F is suspected. Beard and co-workers (1) have shown that the delay of the FA curve is corrected following repair of the coarcted segment.

The theoretical concepts presented above fit the observed differences in human studies and receive support from cinefluorographic studies which have demonstrated the transformation of a normal brachial artery flow pattern to a "regurgitant" and almost stagnant flow pattern by local vasoconstrictive reactions. Model studies clearly show that, if there is no disproportion of U/F allowed in the analog, the curve is nearly identical in all sites measured and the effects of laminar flow cannot be discerned. These methods of investigation also yielded results compatible with the theory that a curve may be altered by slow movement of a vascular column containing several different concentration levels where increased local mixing is possible. All of these are consequences of the increased U/F ratio of that segment.

Although it has been convenient to consider certain portions of the peripheral arterial tree as extensions of "sampling system dead space" (2, 9), it is obvious from Table I that the effects of this type of dead space are quite variable and a single curve could not be analyzed by the approaches which Sherman, Schlant, Kraus and Moore (6) have applied to catheter sampling systems. For this reason, it is suggested that in the consideration of possible alteration of dilution curves only the predictable, measurable invariant "dead space" be used in corrective manipulation.

SUMMARY

Of all commonly used arterial sampling sites, the femoral artery is most likely to yield a curve which most closely approaches the curve as it emerges from the left ventricle and this curve will be recorded with the least delay. Variable alteration of one or more curve parameters was frequently seen when simultaneous brachial artery or radial artery curves were compared with those from the femoral artery. The distortion encountered may assume proportions which would lead to serious inaccuracy in the estimation of appearance time, mean circulation time and disappearance slope.

Radiopaque injections into the femoral and brachial arteries of human subjects without arterial disease show extremely variable flow patterns in the brachial artery but not in the femoral artery. This is apparently due to local vasoconstriction.

A simple branched model characterized by pulsatile flow in an elastic system, with dimensions of capacity and stroke volume proportional to those of the human large arterial bed, could duplicate all variations of dilution curves seen in the human

studies by means of altered resistance to flow in the two branches.

In the absence of pulsatile, elastic flow in the model, alteration of the curves was not seen, thus indicating that the parameters measured were insensitive to the effects of laminar flow and longitudinal mixing.

It is concluded that whenever possible the femoral artery in humans should be the most distal sampling site used when accurate reproduction of the contour of the curve and accurate estimation of the arterial volume included in the "Stewart" or "needle to needle" volume is required.

A high degree of coarctation of the aorta and presumably obstructive disease of the great vessels or aneurysmal dilatation of the thoracic or abdominal aorta render the femoral artery curve invalid for accurate applications of a dilution curve.

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GAS CHROMATOGRAPHIC ANALYSIS OF THE FATTY ACID COMPOSITION OF THE PLASMA LIPIDS IN NORMAL AND DIABETIC SUBJECTS *

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Epidemiological, clinical-pathological, hormonal and nutritional studies during the last ten years indicate that disturbances of the lipid metabolism are important factors in the pathogenesis of atherosclerosis. Increased serum lipid level, induced by nutritional and pathological conditions, is correlated with an increased frequency of atherosclerosis. In diabetic acidosis, hyperlipemia (1–3) with a pronounced increase of free fatty acids (FFA) (4) is regularly found. After treatment with insulin and diet a normal serum lipid level usually occurs in patients with diabetes of short duration, although isolated increase of FFA often is found (5). Even in well controlled diabetic patients an elevation of the serum lipids is often seen when the diabetes has lasted for some years. Adlersberg and co-workers (6) reported that diabetic patients with vascular complications had significantly higher serum lipid levels than did cases with uncomplicated diabetes or normal controls. Lowy and Barach (7) found that this augmentation of the lipids preceded the symptoms of vascular complications.

In the present study a detailed analysis of the fatty acid composition of the different plasma lipid fractions in the postabsorbtive state was performed in seven diabetic patients and in eight healthy individuals used as controls.

MATERIALS AND METHODS

Six males with juvenile diabetes of varying duration, with diabetic vascular complications in different organs, were selected for this study. Later, one woman, who on several occasions had been found to have a high serum lipid level, i.e., 1,200 to 3,000 mg per 100 ml of plasma, was included, although her diabetes had started in adult life. Signs of diseases other than diabetes were not present. The basic diet was conventional Swedish food (excluding sugar, sweet fruits, and food with a high sugar content) with a high supply of protein and moderate restriction of bread and potatoes. Forty to 50 per cent of the total caloric intake was fat, most of it from milk, Swedish margarine (with a high content of coconut oil and rapeseed oil), pork and beef, with only a small part from fish and vegetables. No supplement of oils rich in polyunsaturated fatty acids was given. As a rule the fasting blood sugar level was below 0.200 g per 100 ml of blood and the daily output of sugar in the urine was between 10 and 30 g. Ketoacidosis was not present during the period of lipid analysis. The patients maintained a constant and normal body weight.

The ocular examination was performed by an ophthalmologist. The cardiovascular examinations included electrocardiography (leads I, II, III, IVR, CR_1, CR_2, CR_4 and CR_7) and X-ray examination of the heart, the thoracic and abdominal aortas, and the pelvic and leg arteries. In Case 3, the patient's history was compatible with coronary artery disease. The ECG was normal when the patient was resting but the exercise tolerance test was indicative of coronary insufficiency at 600 kilopond meters per minute. Signs of peripheral vascular insufficiency were not present in the patients, and oscillometric examinations of the extremities gave normal results.

When proteinuria developed during the disease it was taken as evidence of diabetic nephropathy, when repeated investigations failed to demonstrate other disease of the kidneys or the urinary tract. Advanced proteinuria and other signs of the nephrotic syndrome were not present.

At the time of lipid analysis all the patients lived their normal life and were capable of doing their normal work. The clinical data are summarized in Table I.

The eight healthy male human subjects studied were medical students and blood donors, 23 to 38 years of age. They lived on a standard Swedish diet with 30 to 40 per cent of the caloric intake as fat, most of it milk, Swedish margarine, pork and beef, with only a small part from fish and vegetables. One of them (B.J.) was a vegetarian. For several years he had endeavored to consume food containing fats with a very high content of polyunsaturated fatty acids.

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TABLE I

Clinical data relative to the diabetics

Patient	Age	Sex	Ht/Wt	Duration of diabetes	Insulin dosage	Plasma creatinine	Diabetic angiopathy
			cm/kg	*yrs*	*IU/day*	*mg/100 ml*	
J.O.E.	20	M	170/70	14	54	0.9	Retinopathy with capillary microaneurysm and retinal hemorrhages. Nephropathy with proteinuria.
A.B.	25	M	170/60	21	44	1.2	Advanced retinopathy with capillary microaneurysm, retinal hemorrhages, exudates and proliferating retinopathy. Angina pectoris. Vascular calcifications (X-ray) in leg vessels.
B.I.	29	M	163/65	28	64	0.9	Retinopathy with capillary microaneurysm.
H.O.S.*	20	M	165/62	18	64	0.8	Nephropathy with proteinuria.
L.O.S.*	20	M	165/58	12	68	0.7	Nephropathy with proteinuria.
Y.R.	42	M	173/69	32	64	1.6	Retinopathy with capillary microaneurysm, retinal hemorrhages and exudates.
G.B.	36	F	171/64	10	32	0.7	Retinopathy with capillary microaneurysm and retinal hemorrhages.

* Uniovular twins.

Experimental procedure

Blood for analysis was taken in the morning after the subjects had fasted for 10 to 12 hours. Blood sampling and preparation of the total lipid extracts were performed as described earlier (8).

Analytical methods. Total fat was determined by evaporation of an aliquot of the lipid extracts and weighing of the residue. Free and total cholesterol were determined according to the method of Zak and associates (9). FFA were determined according to the method of Svanborg and Svennerholm (8). The concentration was calculated as milligrams of oleic acid per 100 ml of plasma.

Glyceride-glycerol was determined as formaldehyde by the chromotropic acid color reaction according to Lambert and Neish (10), using the modification described by Carlson and Wadström (11). However, ethanol used in the saponification was found to give high and varying blank values. We therefore tried methanol, which was found to give low and constant blank values. From total lipid extracts the phospholipids were removed by chromatography on silicic acid (8). Recrystallized tripalmitin (B.D.H.) was used as standard and the triglyceride value was calculated as tripalmitin. Lipid phosphorus was determined according to Svanborg and Svennerholm (12).

Chromatographic separation of lipids. The plasma lipid classes were separated by the use of silicic acid chromatography as described by Hirsch and Ahrens (13). Silicic acid (Baker AR, lot no. 4680) was milled in a ball mill and screened. Particles passing a 120 DIN sieve were used. In order to remove extremely small particles the silicic acid was then suspended in methanol. After 5 minutes of settling, the methanol was decanted. This procedure was repeated once with methanol and twice with diethyl ether. The remaining silicic acid was dried in air and finally activated at 120° C for 24 hours. Sixty g of the silicic acid powder was suspended in ether and rapidly transferred to a glass column (Figure 1). The silicic acid column, which was about 18 cm in height, was washed with 100 ml of acetone-ethyl ether (1 : 1, vol/vol), followed by 100 ml of ether. Complete removal of the dehydrating solvents was then achieved by washing the column with 1 L of light petroleum (bp 60 to 80° C) for about 12 hours. A 60 g column was charged with 1 g of lipid. The mixture of lipids to be separated was dissolved in a small volume of light petroleum and pipetted on the column. Elution was carried out with solvent mixtures of increasing polarity, as is shown in Figure 2. The flow rate of the solvents was regulated to 2.5 ml per minute with the stopcock at the lower end of the column. Fractions of the eluate, 25 ml each, were collected. One-half of each fraction was used for gravimetric determination and colorimetric or titrimetric analysis (cholesterol, glycerol, phosphorus and FFA); the other half was used for gas chromatographic analysis of the fatty acids.

At the beginning of this investigation the possibility of using the same column in several separations was tested. However, the adsorption power of the silicic acid was found to decrease rapidly. Chromatography with standardized stepwise elution will not give reproducible results if the columns are used more than once.

Hydrolysis and esterification. The cholesterol esters and the triglycerides were hydrolyzed in 0.5 N ethanolic potassium hydroxide (10 ml for 100 mg lipid) by boiling under reflux for 2 to 3 hours. The reaction mixture was diluted with half the volume of water, and the unsaponifiable material was extracted with light petroleum. After

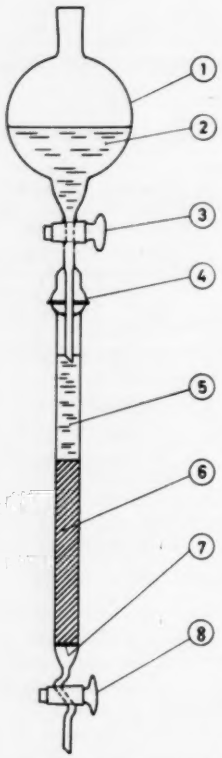

FIG. 1. GLASS APPARATUS USED FOR COLUMN CHROMATOGRAPHY WITH SILICIC ACID. 1 = two L separatory funnel; 2 and 5 = solvent; 3 and 8 = stopcocks; 4 = ball joint; 6 = silicic acid; 7 = coarsely sintered glass filter.

acidification the FFA were extracted from the ethanol-water phase with light petroleum. The solvent was evaporated and the fatty acids were converted into methyl esters by boiling with methanol containing 2 per cent (vol/vol) concentrated H_2SO_4 for 2 hours. (Too great a concentration of H_2SO_4 may lead to changes of the unsaturated acids.) The mixture was diluted with half the volume of water and the methyl esters were extracted with light petroleum. The light petroleum solution was washed with a sodium carbonate-bicarbonate buffer, pH 10, and water. The gas chromatographic analysis of a synthetic mixture of methyl stearate and methyl linolenate (stearate 61.5 per cent, linolenate 38.5 per cent) before and after hydrolysis and re-esterification (stearate 61.2 per cent, linolenate 38.8 per cent) showed that the treatment did not change the polyunsaturated fatty acids. In the first analyses the phospholipids were hydrolyzed and esterified by this method. In order to get a more complete conversion of the sphingomyelin fatty acids to methyl esters the phospholipids were boiled under reflux for 2 hours with methanol containing 5 per cent concentrated H_2SO_4. This procedure had been found to give complete methanolysis of brain cerebrosides. The relatively short reaction time was chosen in order to avoid changes in the polyunsaturated fatty acids. In one case both alkaline hydrolysis and methanolysis were used (see Table VI, "Blood plasma from 3 blood donors"). The principal difference is the higher amount of C_{24} acids split off from sphingomyelin by methanolysis than by alkaline hydrolysis. The proportion between linoleate and oleate is the same after methanolysis of the phospholipids as it is after alkaline hydrolysis, showing that the linoleate is not destroyed during methanolysis. This comparison can be made because the oleic and linoleic acids constitute only 1 per cent each of the sphingomyelin fatty acids.

After the completion of this investigation, however, it became obvious that the content of C_{24} acids was lower than could be expected from the sphingomyelin concentration in the phospholipids. In order to get more detailed information of the efficiency of the hydrolysis and methanolysis methods used, sphingomyelin prepared from human serum was subjected to alkaline hydrolysis and methanolysis. A mixture of 50 mg of sphingomyelin and 5 mg of heneicosanoic acid was boiled in 0.5 N ethanolic potassium hydroxide solution for 2 hours or in 5 per cent concentrated H_2SO_4 in methanol for 1, 2, 4 and 8 hours. The release of fatty acids from sphingomyelin was studied by gas chromatography using heneicosanoic acid as internal standard. During alkaline hydrolysis 20 to 25 per cent of the fatty acids were split off. Methanolysis for 1, 2, 4 and 8 hours split off 33, 50, 75 and 92 per cent of the fatty acids, respectively. The mean molecular weight of the fatty acids in human plasma sphingomyelin was found to be 326. Thus the fatty acids calculated as methyl esters should amount to 44 per cent of the sphingomyelin if the methanolysis is complete. The sphingomyelin constituted about 20 per cent of the phospholipids in serum. Since the sphingomyelin fatty acids amount to only about 10 per cent of the total phospholipid fatty acids, about 92 to 95 per cent of these acids are included in the present gas chromatographic analysis.

Gas chromatography. For gas chromatography (14, 15) Perkin-Elmer gas fractometers with thermistor thermal-conductivity cells as detectors were used. A Hewlett-Packard 425 A, direct current amplifier was used to amplify the detector voltage before recording. The outlet system of the apparatus had been modified to permit collection of fractions of substances with a high boiling point. For a good separation of substances with a high molecular weight it was necessary to increase the temperature of the injection block to about 30 to 40° C above the temperature of the column. The mixtures of the methyl esters of the fatty acids were analyzed on two stationary phases, viz., silicone (Dow-Corning high vacuum grease) and Reoplex 400 (Geigy) (16, 17). The silicone column packing consisted of 30 parts by weight of stationary phase to 100 parts of solid support (Sil-O-Cel, 50 to 75 mesh) which had been pretreated with acid and alkali and carefully washed with water. Before packing, the silicone column filling was heated at 330° C for 1 week in a slow stream of nitrogen (18). The silicone column (3 m) had about 2,800 theoretical plates for methyl stearate. Celite, 80 to 100 mesh, pretreated in

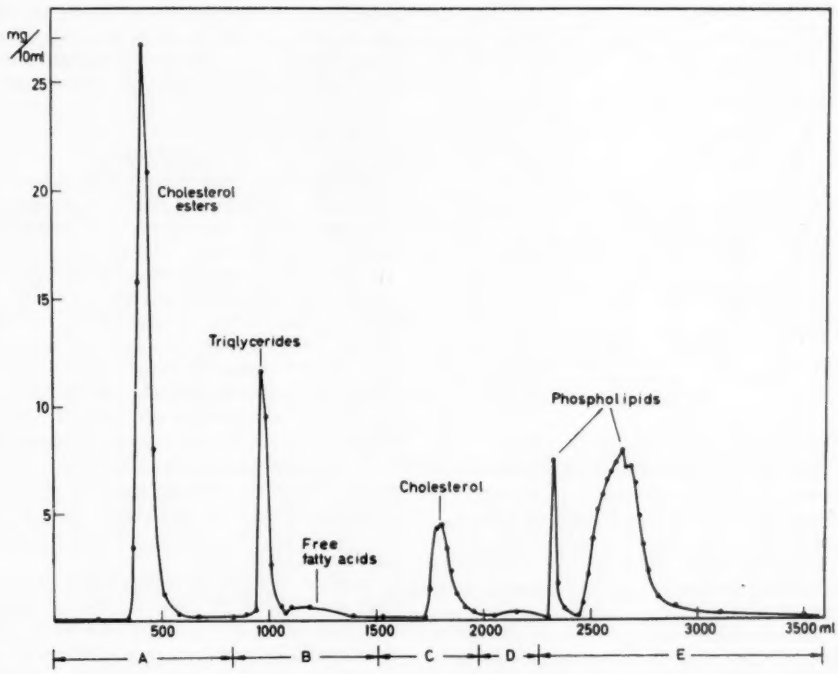

FIG. 2. THE SEPARATION OF THE PLASMA LIPID COMPONENTS BY SILICIC ACID CHROMATOGRAPHY. The following solvents were used: A, light petroleum, bp 60 to 85° C with 1 per cent diethyl ether; B, light petroleum with 8 per cent diethyl ether; C, light petroleum with 25 per cent diethyl ether; D, diethyl ether; E, methanol.

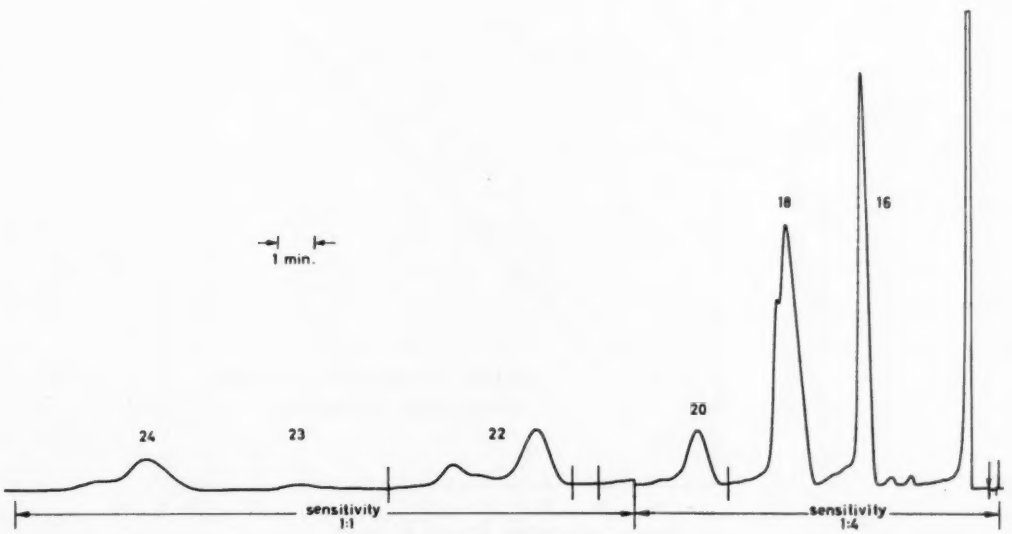

FIG. 3. GAS CHROMATOGRAM ON SILICONE OF THE FATTY ACID METHYL ESTERS OBTAINED FROM PLASMA PHOSPHOLIPIDS. The figures at the peaks denote the number of carbon atoms in the fatty acids. The methyl esters of the C_{18} acids are separated into two peaks, the first representing polyunsaturated acids, the second representing monounsaturated and saturated acids.

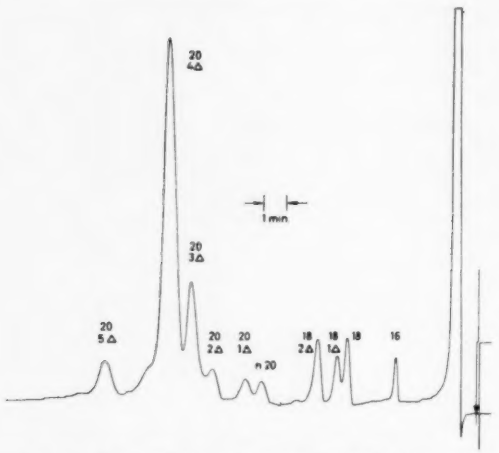

FIG. 4. GAS CHROMATOGRAM ON REOPLEX 400 OF THE METHYL ESTERS OF THE C_{20} FATTY ACIDS (COLLECTED BETWEEN THE SHORT VERTICAL LINES SHOWN IN FIGURE 3) FROM PHOSPHOLIPIDS. 1 △, 2 △, 3 △ and so forth, denote the number of double bonds. The esters of the C_{20} acids are contaminated with traces of those of C_{16} and C_{18} acids.

the same way as Sil-O-Cel was used as supporting medium in the Reoplex column. The same proportion by weight between stationary phase and solid support was used as that in the silicone packing. The Reoplex columns were preconditioned by heating at 240° C in a stream of helium (60 to 80 ml per minute) for at least 20 hours in order to minimize the bleeding and to shorten the retention times. The Reoplex column used (2 m) had 2,500 to 3,000 theoretical plates (for methyl stearate). On silicone the methyl esters separate principally according to the chain length, although the unsaturated esters have a shorter retention time than the saturated ones (Figure 3). Reoplex 400 gives a good separation of the unsaturated esters, the retention time increasing with the number of double bonds. Fractions containing acids of the same chain length were collected from the silicone column and injected into another apparatus containing a Reoplex column. The composition of the mixtures was calculated from the silicone curves and from the fractions analyzed on Reoplex (Figures 3 and 4). For identification of the peaks, logarithmic retention diagrams of the type shown in Figure 5 were used. As can be seen in this figure the methyl ester of eicosapentaenoic acid and docosenoic acid have the same retention time. The same is true for methyl docosapentaenoate and methyl tetracosenoate. These esters, however, separate on silicone. This separation can now be performed on polyesters other than Reoplex (17, 19).

Synthetic mixtures of methyl esters of saturated fatty acids from C_{16} to C_{22} were analyzed on silicone. There was good agreement between the observed values and the theoretical weight percentage composition (see Table II). Methyl esters of the fatty acids obtained from plasma cholesterol esters, triglycerides and phospholipids were

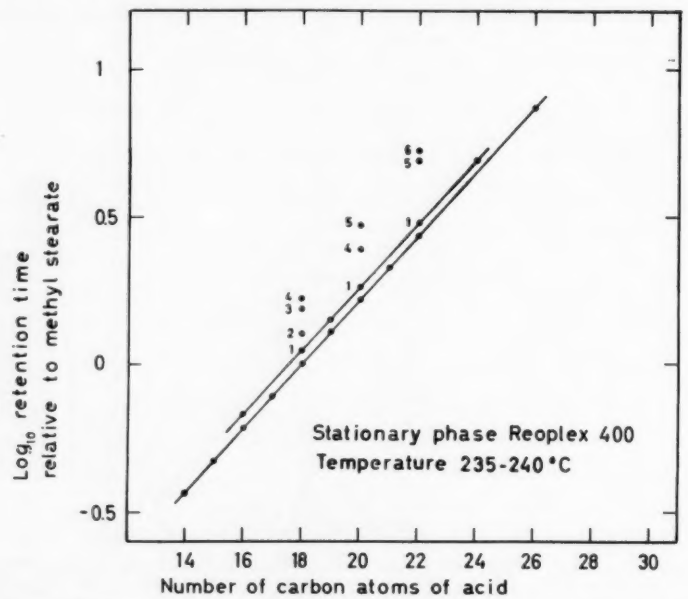

FIG. 5. RELATIONSHIPS BETWEEN THE NUMBER OF CARBON ATOMS OF THE FATTY ACIDS AND LOG_{10} RETENTION TIME RELATIVE TO METHYL STEARATE ON REOPLEX 400 AT 235 TO 240° C. The lower line represents esters of saturated straight chain fatty acids and the upper line represents monounsaturated esters. The figures denote the number of double bonds.

TABLE II

Gas chromatographic analysis on silicone of a synthetic mixture with approximately the same composition as the hydrogenated fatty acids from phospholipids (percentage composition)

	Observed values				Theoretical composition
	3 Repeated injections			Mean	
C_{16}	29.0	30.0	29.6	29.5	28.9
C_{18}	51.3	50.8	50.5	50.9	51.3
C_{20}	10.3	12.0	12.1	11.5	12.4
C_{22}	7.2	7.2	7.8	7.4	7.4

separated on silicone before and after hydrogenation. As can be seen in Table III, somewhat lower figures were found for the C_{20} and C_{22} fatty acids before hydrogenation, indicating a partial loss of the unsaturated acids. When the fatty acid composition was calculated from gas chromatographic analyses on silicone without hydrogenation, correction factors for the C_{20} and C_{22} acids amounting to 10 and 15 per cent of the found values, respectively, were used. These correction factors have been evaluated from many experiments. When mixtures of methyl stearate and methyl linoleate were analyzed on Reoplex columns, 98 per cent of the linoleate was recovered. We used a Reoplex column with short retention times to minimize the loss of polyunsaturated C_{20} and C_{22} fatty acids. To achieve a better separation, the C_{18} fatty acid methyl esters were analyzed at a lower temperature, 200 to 210° C.

TABLE III

Gas chromatographic analysis of plasma lipid fatty acids on a silicone column before and after hydrogenation (percentage composition)

	Cholesterol esters		Triglycerides		Phospholipids	
	Before	After	Before	After	Before	After
C_{12}			1.0	0.6		
C_{14}	2.1	1.5	3.9	3.4	1.0	1.1
C_{16}	15.2	15.4	28.9	28.6	31.9	30.2
C_{18}	75.7	75.2	60.3	60.3	46.8	46.6
C_{20}	6.5	6.9	3.4	3.8	11.7	13.0
C_{22}	0.6	1.0	2.6	3.3	6.1	7.2
C_{23}					0.2	0.4
C_{24}					2.3	2.1

RESULTS

The plasma lipid levels of the diabetic patients and the control subjects are given in Table IV. A typical elution curve from a separation on silicic acid is shown in Figure 2. A small peak of yellow material, probably carotene, appeared first. The colorimetric determinations showed that there were no glycerides in the cholesterol ester peak, neither was there any cholesterol in the triglycerides. In the cholesterol esters and in the triglyceride fraction the more saturated components were found to come first and the more unsaturated ones were concentrated in the tail of the peaks. Among

TABLE IV

Plasma lipid levels of normal control subjects and of patients with diabetes

Subjects	Age	Sex	Total lipids	Cholesterol		Phospholipids	Triglycerides	Free fatty acids
				Total	Free			
			mg/100 ml	*mg/100 ml*		*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
Normal cases:								
W.J.M.	38	M	640	204	58	170	121	17.0
L.H.	28	M	706	282	90	221	61	13.7
E.H.	23	M	591	204	62	209	58	16.8
Blood plasma from 3 blood donors	23	M	527	212	64	217	113	19.7
K.H.H.	23	M	474	217	72	168	29	28.0
Mean			588	224	69	197	76	19.0
Vegetarian:								
B.J.	26	M	424	128	43	150	76	19.0
Diabetics:								
J.O.E.	20	M	1,233	305	96	352	390	26.6
A.B.	25	M	1,120	438	142	343	98	48.0
B.J.	29	M	830	206	85	300	179	36.7
H.O.S.	20	M	520	141	62	212	62	11.8
L.O.S.	20	M	600	209	107	235	68	26.3
G.B.	36	F	1,145	275	113	355	318	43.7
Y.R.	42	M	942	387	109	323	99	32.4
Mean			913	280	102	303	173	32.2

TABLE V
Fatty acid composition of cholesterol esters

Subjects	C_{14}	C_{16}	C_{16} sat.	C_{16} monoene	C_{17}	C_{18} sat.	C_{18} monoene	C_{18} diene	C_{18} triene	C_{20} tetraene	C_{20} pentaene	C_{22} pentaene + hexaene
Normal cases:												
W.J.M.	1.3	0.3	11.6	5.9	0.8	0.8	22.9	44.5	1.3	6.7	2.9	0.4
L.H.	1.5	0.5	10.4	4.5	1.0	1.1	22.7	48.6	1.6	5.3	1.4	0.8
E.H.	1.6	0.8	11.4	4.2	1.2	0.7	20.2	45.6	3.9	6.3	6.3	0.6
Blood plasma from 3 blood donors	1.3	0.7	10.6	4.3	0.9	0.9	21.3	47.9	1.4	6.3	2.1	0.5
K.H.H.	1.1	0.6	11.1	5.2	1.7	0.6	28.9	44.7		4.7	0.7	0.5
Mean	1.4	0.6	11.0	4.8	1.1	0.8	23.2	46.2	2.1	5.9	1.8	0.6
Vegetarian:												
B.J.	0.6	0.3	9.3	1.8	0.5	0.8	18.5	61.3		5.3	0.5	0.4
Diabetics:												
J.O.E.	1.2	0.3	10.4	5.5	1.3	0.8	26.0	45.9	3.1	4.4	0.6	
A.B.	0.9	0.4	11.4	3.2	1.3	0.7	18.0	52.6	1.9	7.5	1.2	0.7
B.J.	1.5	0.3	13.3	5.1	0.9	0.2	25.4	44.2	1.1	4.1	2.5	0.8
H.O.S.	0.9	0.5	9.8	6.5	1.4	1.0	24.6	43.7	2.8	4.0	1.6	
L.O.S.	1.2	0.4	11.8	2.7	0.9	1.0	24.4	50.6		5.4	0.8	0.4
G.B.	0.9	0.4	13.5	3.5	1.0	1.0	24.8	40.2	1.4	8.0	3.7	1.0
Y.R.												
Mean	1.1	0.4	11.7	4.4	1.1	0.8	23.9	46.2	2.1	5.6	1.7	0.7

the saturated esters, those with long chains were found to move faster than those with shorter chains.

Immediately after the triglycerides followed a small peak containing lipids with titratable acidity, the free fatty acids. The next peak, eluted by 25 per cent of diethyl ether in light petroleum, contained free cholesterol. Sometimes two small peaks giving glycerol reaction surrounded the cholesterol peak. The first one was probably diglyceride and the second monoglyceride, as described by Hirsch and Ahrens (13). Upon addition of methanol, the cephalins leave the column in the first peak. This peak also contains phosphorus free lipids, viz., cerebrosides and sulfatides, as could be demonstrated by paper chromatography (20). The second large peak consisted mainly of lecithin and sphingomyelin. The sphingomyelin and lysolecithin were found in the tail of the peak. This could be established by paper chromatography of the lipids and by analysis of the fatty acids for sphingomyelin.

The results of the plasma fatty acid analysis in the present investigation are shown in Tables V, VI, and VII. It can be seen that no definite difference between healthy individuals and diabetics on comparable diets was found. On the contrary, there was a remarkable similarity between the two groups. Striking differences were noted, however, in the levels of the various lipid fractions, as can be seen in Table IV. The diabetic group had a significant increase in the total lipid, cholesterol, phospholipid, triglyceride and FFA fractions.

One of the healthy individuals studied (B.J.) had been a vegetarian for several years. During the last six months he had included 40 to 60 g of corn oil daily in his diet. Among the different plasma lipid fractions, the highest increase of linoleic acid was found in the triglycerides. Linoleic acid constituted 39 per cent of the triglyceride fatty acids in this case as compared to 11 per cent in the other healthy individuals (Table VI). In the cholesterol esters the increase was from 46 to 61 per cent and in the phospholipids from 21 to 29 per cent. Our results thus confirm the findings of Ahrens and co-workers (21) that the phospholipids appear to have the most constant fatty acid pattern of any serum lipid group. The triglycerides and cholesterol esters more strongly reflect the changes in dietary fats.

The results of the fatty acid analysis in the different plasma lipid fractions have been used for studying the validity of the conventionally used factors for calculation of the amount of cholesterol esters from cholesterol determination, triglycerides

TABLE VI

Fatty acid composition of triglycerides

Subjects	C_{14}	C_{15}	C_{16} sat.	C_{16} monoene	C_{17}	C_{18} sat.	C_{18} monoene	C_{18} diene	C_{20} monoene	C_{20} tetraene	C_{20} pentaene	C_{22} pentaene	C_{22} hexaene
Normal cases:													
W.J.M.	2.3	0.4	30.0	4.6	1.2	4.4	40.3	11.4	1.0	1.0	0.2	0.5	1.0
L.H.	3.5	1.1	20.4	17.1	2.0	4.7	40.0	12.8	1.7	1.0	0.7	0.7	1.7
E.H.	3.1	1.0	24.8	8.2	2.2	3.1	39.3	12.0	0.9	0.7	0.8		
Blood plasma from 3 blood donors	4.2	1.4	25.0	5.3	2.0	4.7	38.2	9.1	1.9	0.8		0.5	0.7
K.H.H.	2.1	0.7	24.3	5.9	1.7	3.1	49.0	9.3	1.6	0.5		←1.0→	
Mean	3.0	0.9	24.9	6.2	1.8	4.0	41.4	10.9	1.4	0.8	0.6	0.6	1.1
Vegetarian:													
B.J.	1.1	0.4	15.2	3.3	0.5	2.0	31.9	39.0	0.6	1.6		0.2	0.1
Diabetics:													
J.O.E.	2.8	1.0	27.7	5.9	1.8	5.1	42.9	8.1	1.0	0.6	0,1	0.4	0.4
A.B.	2.1	0.4	21.5	5.0	4.6	5.8	39.6	13.9	1.2	1.1		0.7	1.2
B.J.	2.3	0.6	26.2	6.9	1.9	4.0	38.2	10.3	1.8	0.9	0.5	0.5	1.6
H.O.S.	3.3	1.3	27.4	6.1	2.7	4.3	40.7	7.0	←	2.6	→	← 1.9 →	
L.O.S.	1.9	0.5	29.4	3.9	1.0	5.1	41.8	11.5	1.5	0.9	0.1	0.5	0.7
G.B.	2.1	1.0	26.6	6.2	1.9	4.7	33.1	14.7	1.1	1.4	0.6	1.0	1.9
Y.R.	2.5	0.6	29.3	2.5	1.2	4.7	38.8	12.1	←	3.5	→	← 3.7 →	
Mean	2.4	0.8	26.9	5.2	2.2	4.8	39.3	11.1	1.3	1.0	0.3	0.6	1.2

TABLE VII

Fatty acid composition of phospholipids

Subjects	C_{14}	C_{15}	C_{16} sat. + monoene	C_{17}	C_{18} sat.	C_{18} monoene	C_{18} diene	C_{20} triene	C_{20} tetraene	C_{20} pentaene	C_{22} pentaene	C_{22} hexaene	C_{24} monoene	
Normal cases:														
W.J.M.	0.5	0.8	29.6	2.1	11.2	13.7	18.0	2.7	10.6	2.6	1.4	5.3		A*
L.H.	0.6	1.0	26.0	2.2	11.8	15.9	20.7	1.8	9.5	1.7	1.1	6.2		A
E.H.	0.5	0.6	24.7	2.0	13.2	14.7	21.2	3.1	9.3	2.0	1.6	4.1		A
Blood plasma from 3 blood donors	0.6	0.4	28.1 27.2	1.1 0.8	9.6 11.5	12.5 11.9	24.3 22.5	2.0 1.9	8.7 7.5	2.2 1.9	0.5 0.7	4.8 3.4	1.4 4.1	A M†
K.H.H.	0.5	0.4	30.9	1.0	11.7	15.3	19.8	2.6	7.0	0.8		4.0	2.9	M
Mean	0.5	0.6	27.7	1.6	11.9	14.3	20.4	2.4	8.8	1.8	1.2	4.8		
Vegetarian:														
B.J.	0.3	0.5	22.8	0.8	15.5	10.0	29.1	3.2	12.4		0.2	2.0	0.9	A
Diabetics:														
J.O.E.	0.5	0.4	30.0	1.5	10.8	19.6	20.1	2.8	8.1	0.8	1.2	2.0	0.4	A
A.B.	0.3	0.7	28.6	1.2	11.1	13.7	23.0	1.4	10.7	0.8	0.8	4.9	0.7	A
B.J.	0.4	0.4	28.7	0.5	12.4	13.8	18.0	1.8	6.4	1.8	1.2	5.9	3.1	M
H.O.S.	0.8	0.6	30.9	1.1	10.8	15.9	20.3	2.7	6.2	2.1	2.2	1.8	2.5	M
L.O.S.	0.3	0.3	28.7	0.8	12.8	13.1	24.2	2.5	7.8	0.9	1.0	2.3	2.0	M
G.B.	0.4	0.3	29.9	1.2	11.4	12.8	16.3	1.8	10.3	2.3	1.1	6.0	2.5	M
Y.R.	0.4	0.5	28.5	1.0	14.8	13.0	18.3	1.9	8.5	0.9	0.4	2.9	3.0	M
Mean	0.4	0.5	29.5	1.1	12.0	14.6	20.0	2.1	8.3	1.4	1.1	3.7	2.6	

* A = alkaline hydrolysis.
† M = methanolysis.

from glycerol, and phospholipids from phosphorus determination. The mean molecular weight of the fatty acids of the cholesterol esters was found to be 276 and of the triglycerides 272. The factor for calculating the phospholipids from phosphorus analysis was checked by comparison with the gravimetric determinations of the phospholipids after chromatographic separation. This factor was found to be 25.

DISCUSSION

In order to avoid cases with senile atherosclerotic vascular disease, juvenile diabetics and one middle-aged patient with adult diabetes were selected for this study. Three of the patients studied had early signs of vascular complications in different organs and four had signs of more advanced complications. When the lipid analyses were performed rapid progression of the vascular complications was present in most of the patients. Therefore, it seems reasonable to assume that changes in the fatty acid composition of the plasma lipids should have been demonstrable in these patients if such changes really were of pathogenic significance in diabetic vascular disease.

The knowledge of the disturbances of the lipid metabolism in diabetes is only fragmentary. It is clear from many lines of research that the synthesis of both cholesterol and fatty acids is greatly influenced by the metabolism of glucose (22, 23). Decreased synthesis both of fatty acids and cholesterol is reported in diabetes and the cause of this lipogenetic defect was recently discussed by Siperstein and Fagan (23). The finding that the FFA in plasma often are abnormally high, even in well controlled diabetic patients (5), indicates an abnormally high mobilization of fatty acids from fat depots in diabetes. As has been mentioned earlier, other plasma lipid fractions also, especially the triglycerides, are often found to be abnormally high in diabetics. According to our experiences, the postabsorptive plasma lipid level shows greater variability from day to day in diabetics than it does in healthy individuals.

Atherosclerosis is a metabolic disease in which altered lipid metabolism seems to play an important role. Numerous investigators have studied the levels of the plasma lipids in individuals with atherosclerosis during middle age and found an elevated plasma lipid level to be a cardinal sign of the disease. The disturbances of the lipid metabolism in diabetes and the tendency to hyperlipemia have been proposed to be the pathogenic factors responsible for the high frequency of atherosclerosis among diabetics.

Dietary fats rich in polyunsaturated fatty acids have been found to decrease the plasma lipid level and to have a prophylactic and therapeutic effect on atherosclerosis in experimental animals (24). Some investigators have therefore proposed that there might be a deficiency of polyunsaturated fatty acids in patients with atherosclerosis. However, in a study of the plasma fatty acid composition of the phospholipids and of the acetone-soluble fraction of plasma lipids, James, Lovelock, Webb and Trotter (25) failed to demonstrate any difference between healthy individuals and patients with ischemic heart disease. In the present investigation of diabetics with vascular complications a more detailed analysis of the fatty acids in the different plasma lipid fractions has been performed. The separate analysis of the triglyceride fraction seems to be of special interest, since this fraction is often elevated in the plasma of diabetics when cholesterol esters and phospholipids lie within normal limits.

The present investigation has demonstrated that diabetics and healthy individuals of the same age, on comparable diets, have the same plasma fatty acid composition. These observations contradict the hypothesis that a deficiency of polyunsaturated fatty acids is an essential factor in the pathogenesis of the vascular complications in diabetes. It is more probable that the increased levels of the different plasma lipid fractions play an important role. The largest percentage increase was found for the triglycerides, which is in accordance with earlier findings (6). The dietary management of diabetes must be directed toward a normalization not only of the blood sugar level but also of the blood lipid level.

SUMMARY

The fatty acid composition of cholesterol esters, triglycerides and phospholipids was studied by gas chromatographic analysis in seven diabetics with vascular complications and in seven healthy individuals of the same age on a similar diet. The fatty acid composition of the different plasma lipids was found to be remarkably similar in the two groups. The only pathological plasma lipid

finding demonstrable in the diabetics was an abnormally high plasma lipid level in some cases.

During chromatogarphy on silicic acid the more saturated cholesterol esters and triglycerides were found to move faster than the more unsaturated ones.

The accuracy of the conventionally used factor of 25 for calculating phospholipids from phosphorus determinations was substantiated. In the average subjects the mean molecular weights of the cholesterol ester fatty acids and triglyceride fatty acids were found to be 276 and 272, respectively.

In an additional study of the plasma lipids of a healthy vegetarian consuming 40 to 60 g of corn oil per day, the most marked increase of linoleic acid was found in the triglycerides along with a smaller increase in the cholesterol esters. The phospholipids were found to have the most constant fatty acid pattern.

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THE INFLUENCE OF CORTISONE ON EXPERIMENTAL FUNGUS INFECTIONS IN MICE *

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The administration of adrenal glucocorticoids is associated with enhancement of many experimental infections. The pertinent studies have been reviewed comprehensively by Kass and Finland (1). Most experiments in which fungi were used as the infecting microorganism have also demonstrated an increase in the severity of infection when adrenal hormones were injected prior to or concomitantly with the fungus (2–9). A few studies, however, showed no augmentation of mycotic disease following the administration of steroids (3, 10–12).

The investigations on fungi have not provided information as to the relative propensity of the various species to disseminate under the influence of steroids, nor have they offered definitive data on the mechanisms of enhancement or on the tissue localization of increased infection.

The effects of cortisone on experimental infections in mice caused by *Histoplasma capsulatum*, *Cryptococcus neoformans*, and *Candida albicans* were compared in the present studies. The results show that Monilia infections were enhanced more readily than infections caused by either of the other two fungi. Further bacteriological and histological studies were then performed to delineate the pathogenesis of increased infection in candidiasis, and to analyze the effects of cortisone when the infecting inoculum was small and when steroid treatment was delayed.

* This work was supported in part by grants from the National Institute of Allergy and Infectious Diseases, Bethesda, Md. (E-2659), the Charles Pfizer Co., Inc., New York, The Health Research Council of New York City, The New York Trudeau Society, and Training Grant E-6 from the National Institute of Allergy and Infectious Diseases. A preliminary report of these studies was presented to the Second Conference on Medical Mycology held under the auspices of The New York Academy of Sciences, January 11 and 12, 1960.

MATERIALS AND METHODS

Mice. Two strains of mice were used. In all studies on histoplasmosis and cryptococcosis, and in some of the Candida experiments, female Swiss albino mice, 17 to 20 g, from the National Institutes of Health (NIH) were used. Mice of this strain tolerated up to 2.0 mg cortisone per day for 28 days without weight loss or other noticeable deleterious effects, although some animals failed to gain weight during steroid administration.

In later experiments, male Swiss albino mice 17 to 20 g, from Carworth Farms (strain CFW) were used. Mice of this strain consistently lost weight when 0.5 mg of cortisone was administered daily for 5 to 9 days. Increase in the dosage of 1.0 mg daily for 9 days produced death in up to 30 per cent of control animals. Experiments on moniliasis using this strain were included in the results after it was determined that death curves and tissue populations were similar to those obtained with NIH mice in both normal and cortisone-treated animals.

Mice of both strains were variably housed in glass jars, or in metal or plastic cages. They were fed mouse pellets and water *ad libitum*.

Fungus strains. Histoplasma capsulatum, strain 6624, was recovered from a patient with disseminated histoplasmosis in 1952. During serial weekly passage at 37° C, on blood-cysteine agar, it has not altered in pathogenicity for mice, killing at least 80 per cent of animals within 30 days following the intravenous inoculation of 5×10^6 yeast phase cells.

Cryptococcus neoformans, strain 1148, was recovered in 1954 from the cerebrospinal fluid of a patient with chronic cryptococcus meningitis. This strain, a large capsule strain, has not altered in pathogenicity for mice during semi-weekly serial passage on Sabouraud's agar slants incubated at 30° C. After intravenous inoculation of 2.5×10^6 cells, 90 to 100 per cent of mice die within 8 weeks with gross hydrocephalus.

Candida albicans, strain 851, was recovered in 1956 from the oropharynx of a patient with clinical evidence of mucocutaneous moniliasis. It has been maintained by serial monthly passage on Sabouraud's agar slants incubated at 30° C. There has been no change in mouse pathogenicity during this period. Five to 30 per cent of mice die within a 4 week period after the intravenous injection of 5×10^6 cells. When the inoculum is

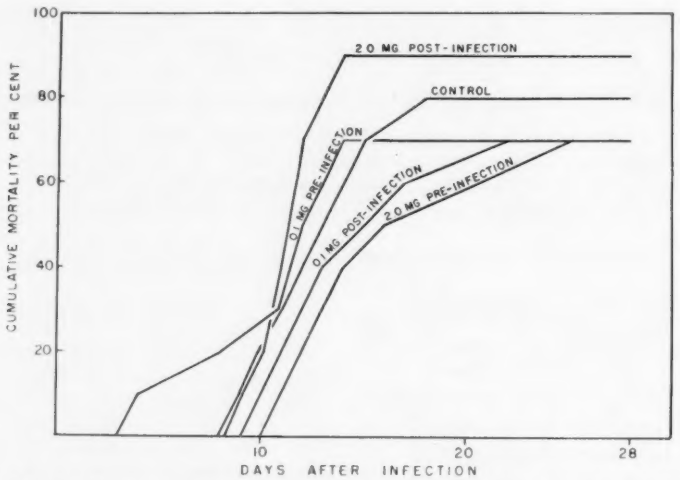

FIG. 1. EFFECT OF CORTISONE ON MORTALITY IN MICE FOLLOWING I. V. INFECTION WITH 1×10^6 CELLS OF *H. CAPSULATUM*. Cortisone was administered intraperitoneally daily starting 6 days prior to (pre-infection) or the day following infection (post-infection) and was continued for 28 days after infection. Each group consisted of ten mice.

increased to 10^7 cells, the majority of mice dies within 7 days of infection.

Infection. The infecting dose of *H. capsulatum* and *C. neoformans* ranged from 3×10^4 to 10^7 cells per mouse in these experiments. The size of the Candida inoculum varied from 10^3 to 5×10^6.

Candida and Cryptococcus inocula were emulsified in physiological saline from a Sabouraud's agar slant incubated for 24 hours at 30° C. Histoplasma inocula were emulsified in saline from a 48 hour cysteine-blood agar slant incubated at 37° C. The inoculum in each case consisted almost exclusively of yeast cells which were single or in pairs.

The size of the larger inocula was determined by direct fungus counts in a Neubauer-Levy hemocytometer. The number of fungus cells in smaller inocula was determined by making Sabouraud's agar pour plates of serial 10- or 100-fold dilutions of the injected suspension. Pour plates were incubated at 30° C and colony counts were made at appropriate intervals.

Cortisone. Cortisone acetate was purchased from Schering Corporation, Bloomfield, N. J. One-tenth to 2.5 mg was injected subcutaneously or intraperitoneally in a 0.2 ml volume. Cortisone was administered beginning 2 to 6 days prior to, or starting the day after infection, and was continued for 7 to 30 days after infection. In studies in which 2.5 mg was administered, however, the steroid was only given the day prior to, the day of, and the day after infection, since more prolonged treatment at this dosage produced toxicity and death.

In two experiments on the effects of delayed treatment, administration of 0.5 mg of cortisone was begun 7 days after the initiation of infection and was continued for 9 days.

In each experiment one group of 10 uninfected mice was given the maximum dose of cortisone used in the experiment to insure that deaths in infected animals could not be attributed to cortisone alone.

Tissue population studies. The number of fungi in tissues was determined in control and cortisone-treated mice on the seventh or fifteenth day after infection. In early experiments, liver and spleen or liver, spleen and kidneys were emulsified together in 5 ml of physiological saline, ground with a mortar and pestle, and serially diluted in distilled water. One ml aliquots of appropriate dilutions were cultured on the surface of Sabouraud's agar pour plates.

Liver and spleen were chosen for studies of Histoplasma because there is clear pathological and bacteriological evidence that these are the organs of maximum census following experimental mouse infection (13, 14).

Liver and spleen were also used for fungus enumeration in Cryptococcus experiments after it was shown that progressive infection occurred in all organs studied including liver-spleen (which were combined), lung, kidney, and brain during the first 2 weeks after infection (15). Subsequently, more detailed quantitative studies in this laboratory have demonstrated that multiplication during the first 2 weeks was somewhat less in the liver-spleen than in the lungs, kidney, and brain. It is possible, therefore, that the use of the combined liver and spleen for fungal census studies in cryptococcosis did not represent the most sensitive index of cortisone dissemination.

The kidney was added to the liver and spleen in the initial studies on moniliasis because there was considerable histological evidence that the kidney is extensively

TABLE I

Effect of cortisone on intravenous fungus infections in mice

Fungus	Inoculum size	Control*	Cortisone (0.1 mg/day)*	Cortisone (0.5–2.0 mg/day)*
Candida albicans	10^4	0/10	0/20	0/20
	10^5	0/20	0/40	1/40
	10^6	3/30	10/60	21/60
	5×10^6	8/70	11/70	59/70
Histoplasma capsulatum	10^4	0/10	2/20	0/20
	10^5	1/10	2/20	4/20
	10^6	8/10	14/20	14/20
	10^7	10/10	10/10	10/10
Cryptococcus neoformans	2×10^4	1/10	3/20	3/20
	2×10^5	7/10	9/20	10/20
	2×10^6	9/10	15/20	14/20

* The ratios given show the relationship between the number of mice killed and the number infected.

involved in normal animals following intravenous Candida infection (16, 17).

In the subsequent studies on the tissue localization of Candida infections, serial determinations were made separately on liver, spleen, heart, lung, kidneys, and brain 15 minutes to 60 days after infection. Organs in these later experiments were homogenized in 2 ml of distilled water in a Teflon tissue homogenizer (18), and Sabouraud's agar pour plates were made of appropriate 100-fold dilutions. All pour plates were incubated at 30° C.

In some experiments in which tissue census was expected to be low, pour plates were made of the original undiluted organ homogenate. Control studies demonstrated that homogenates of normal mouse organs had no fungicidal effect on the growth of Candida. Thus, when known numbers of fungi were added to the homogenate, mixed thoroughly and enumerated by making pour plates of the mixture, the same number of fungi was recovered as was inoculated initially into the tissue suspension.

Blood cultures. Mice were anesthetized with ether and bled through the axillary artery. One ml of blood was inoculated onto the surface of 20 ml of Sabouraud's agar in a 250 ml flask. Colony counts were made at 7 and 14 days.

Observation period. When groups of mice were inoculated with 10^4 to 10^7 cells for determination of mortality rates, animals were observed for 26 to 40 days after initiation of infection.

Histological studies. Mice were sacrificed 30 minutes to 30 days after intravenous infection with 10^5 to 10^7 cells of *C. albicans*. Sections of brain, lung, heart, liver, spleen, adrenal, kidney, pancreas and skeletal muscle were studied. These were stained with Masson's trichrome stain or by the periodic acid-Schiff (PAS) technique.

Touch preparations of kidney, liver, lung and muscle

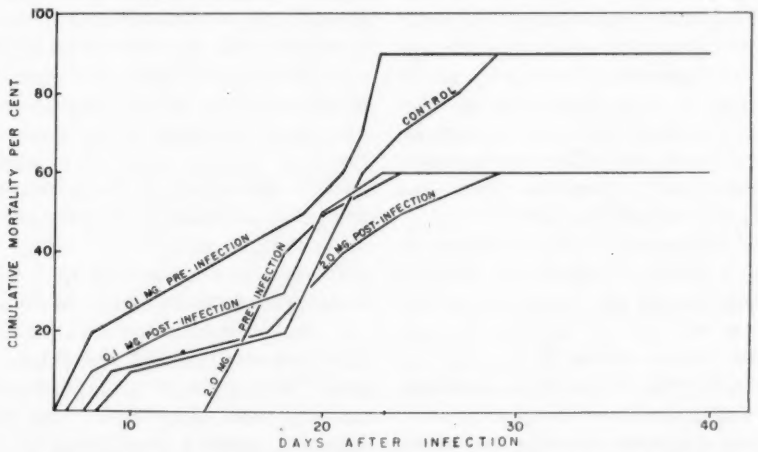

FIG. 2. EFFECT OF CORTISONE ON MORTALITY IN MICE FOLLOWING I. V. INFECTION WITH 2×10^6 CELLS OF *C. NEOFORMANS*. Cortisone was administered intraperitoneally daily starting 6 days prior to (pre-infection) or the day following infection (post-infection) and was continued for 28 days after infection.

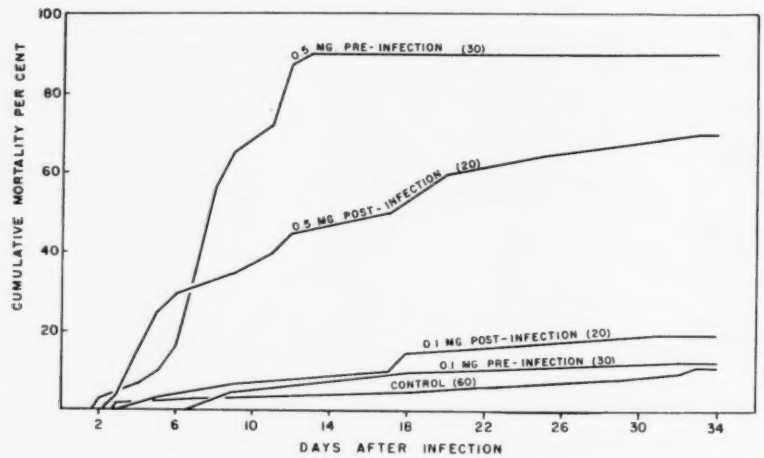

FIG. 3. EFFECT OF CORTISONE ON MORTALITY IN MICE FOLLOWING I. V. INFECTION WITH 5×10^5 CELLS OF *C. ALBICANS*. Cortisone was administered subcutaneously daily for nine days beginning two days prior to (pre-infection) or the day following infection (post-infection). Number of mice in each group is shown in parentheses.

lesions were made and were stained with Wright's stain or by the PAS technique.

RESULTS

Histoplasma capsulatum. The administration of 0.1 to 2.0 mg daily of cortisone starting six days prior to or the day following infection did not alter the mortality or speed of death in mice infected with 10^4 to 10^7 cells of *H. capsulatum*.

Liver-spleen populations were studied in groups of 15 mice 15 days after infection with 3×10^1 cells. These were generally similar in cortisone-treated and control animals. These findings are depicted in Figure 1 which shows the mortality rates in an experiment in which animals were infected with the 10^6 inoculum. The total mortality at each inoculum size in cortisone-treated and control mice is summarized in Table I.

Cryptococcus neoformans. No differences in mortality rates or rapidity of death were observed between cortisone-treated and control groups following infection with 2×10^4 to 2×10^6 cells. Liver-spleen census was studied 15 days after infection with 3×10^2 cells. Populations were similar in control and in cortisone-treated animals.

These findings are recorded in Figure 2 which depicts the results of an experiment in which animals were infected with an inoculum of 2×10^6 cells. The total mortality at each inoculum size in cortisone-treated and control mice is included in Table I.

Candida albicans. No increase in mortality was observed in cortisone-treated animals after infection with 10^4 or 10^5 cells. When the inoculum was increased to 10^6, however, marked modification of the course of the infection was noted in the steroid-treated mice. Three of 30 controls (10 per cent) died during the 30 day observation period. Seven of 40 (17.5 per cent) which were given 0.1 mg of cortisone daily died. Twenty-one of 60 mice (42 per cent) given 0.5 to 2.0 mg of cortisone beginning prior to or one day following infection died during the observation period. The percentage of deaths in the group treated with the large dose of cortisone is significantly different statistically from the death rate in the control group according to the method of chi square ($p = < 0.05$).

Increase in the inoculum to 5×10^6 produced more striking results. Eight of 70 controls (11.4 per cent) died of infection as did 11 of 70 mice (15.7 per cent) treated with 0.1 mg of cortisone daily. In contrast, 59 of 70 (82.9 per cent) given the large dose of cortisone prior to or one day following infection died during the experimental period.

The experiments employing an inoculum of 5×10^6 cells per mouse are summarized in Figure

3. It may be seen that the mortality rate was 90 per cent in mice given 0.5 mg of cortisone two days prior to infection as compared to 70 per cent in animals in which treatment was begun one day after infection. These differences, however, are not statistically significant.

The over-all mortality observed during the experimental period at each inoculum size in normal and in steroid-treated animals is recorded in Table I.

The results of studies on the combined liver-spleen-kidney populations of mice infected with 10^5 cells supported the experiments on gross mortality rates.

Twenty-one of 25 control mice had small tissue populations at the time of sacrifice ranging from 0 to 3.0×10^2 per ml. In the other four, fungus census was between 10^4 and 10^5 per ml. Tissue census was not significantly different in mice given 0.1 mg of cortisone.

In contrast, 24 of 25 mice given 0.5 to 2.0 mg of cortisone had tissue fungus census which was greater than 10^5 per ml, and which in most titers was greater than 10^6 per ml. The differences between tissue populations in control mice and animals given the large dose of cortisone are highly significant ($p = < 0.01$).

Thus, the administration of large amounts of cortisone produced a marked increase in the mortality and tissue census following infection with *C. albicans* but did not enhance infections due to *H. capsulatum* or *C. neoformans*. The studies summarized below were then performed to delineate the site of maximum Candida infection in normal mice and to determine the effects of steroid administration on Monilia populations in the various organs.

Tissue census studies in normal mice infected with 10^5 to 5×10^6 cells of C. albicans. Kidneys were studied in 186 animals, liver in 77, lungs in 49, heart in 45, brain in 38 and spleen in 20. The kidney was the only organ in which progressive fungus multiplication was observed.

When 10^5 cells were injected, kidney populations ranged from 8×10^2 to 5.3×10^3 15 minutes after inoculation. No significant change was observed during the next 24 hours. Kidney fungus census increased more than tenfold above 2 standard deviations from the mean observed 15 minutes to 24 hours after infection in 8 of 56 mice, or approximately 14 per cent studied over the next 30 days. However, a progressive decrease was observed in the number of fungi recovered from the kidneys of most mice. In 13 of 21 animals studied 14 to 30 days after the initiation of infection no Candida was detected in renal homogenates.

When the inoculum was increased to 5×10^6 cells, kidney populations were somewhat higher initially, ranging from 5.8×10^4 to 1.6×10^5. Approximately 11 per cent of normal mice which were infected with this inoculum died, and in these animals there was evidence of progressive renal infection. In 7 of 61 survivors (11.5 per cent), sacrificed 7 to 30 days after inoculation, the number of Monilia in the kidneys had increased more than tenfold above 2 standard deviations from the mean census observed during the first 24 hours. Renal Candida populations in the other 54 animals tended to remain stationary during the first two weeks of infection, then fell in some animals so that 12 of 26 examined 30 days after infection demonstrated virtual clearance of the infection from the kidneys.

Bacteriological events in the other tissues studied were considerably different. Fungus populations were approximately tenfold greater in the liver immediately after infection. Monilia census did not change significantly in the liver, spleen, heart, lungs and brain during the initial 24 hours after inoculation, but then fell progressively in each of these organs, so that one week after infection populations were almost invariably small both in mice infected with 1×10^5 cells and in those inoculated with 5×10^6 cells. No fungi could be detected in these organs two weeks after infection.

In summary, there was evidence of progression of infection within the kidneys in 14 to 22.5 per cent of mice infected with 1×10^5 to 5×10^6 cells. No increase in tissue census was observed in the other organs studied. Clearance of the infection was also slower in the kidneys than in the other tissues examined.

Tissue populations in cortisone-treated mice infected with 1×10^5 cells of C. albicans. Cortisone was administered subcutaneously in daily dosage of 0.5 mg beginning two days prior to infection and was continued for six days after the initiation of infection. The inoculum size of 10^5 cells was chosen because it was the largest inoculum at

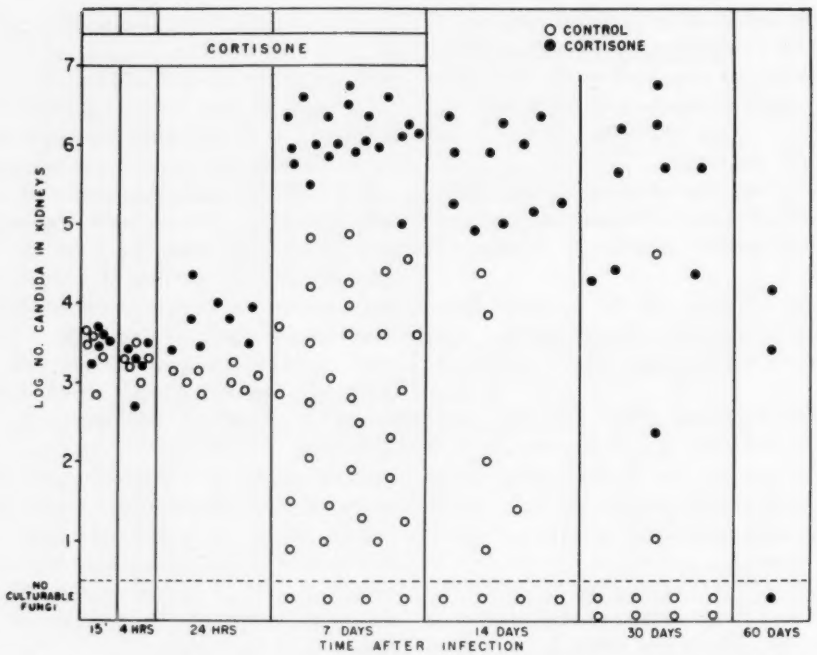

FIG. 4. THE EFFECT OF CORTISONE ON MOUSE KIDNEY POPULATIONS FOLLOWING INTRAVENOUS INFECTION WITH 10^5 CELLS OF *C. ALBICANS*. Cortisone was administered subcutaneously daily in dosage of 0.5 mg starting two days prior to infection.

which deaths from cortisone enhancement were rare. Thirty-day tissue population studies could, therefore, be performed readily.

Kidney populations were determined in 90 mice treated with cortisone over the 30 day period. Renal census was similar in control and in cortisone-treated mice 15 minutes to 4 hours after inoculation, and ranged from 5×10^2 to 5.2×10^3. Twenty-four hours after infection, populations were generally somewhat larger in steroid-treated mice. These differences are statistically significant at the 5 per cent level. Thereafter, cortisone-treated mice invariably demonstrated marked increase of infection within the kidneys. At the termination of cortisone treatment renal census ranged from 1×10^5 to 4.1×10^6. Kidney titers remained high seven days after stopping cortisone in all mice studied but diminished in four of ten animals examined 23 days after stopping steroid treatment.

These findings stand in striking contrast to the kidney populations observed in control mice. As indicated above, increasing kidney titers were observed in only 14 per cent of animals infected with 1×10^5 cells and in most mice there was a progressive decrease in the numbers of Monilia in the kidneys. These studies are summarized in Figure 4.

Bacteriological events were markedly different in the liver, lungs, heart, spleen and brain. Populations were similar in cortisone-treated and control mice 15 minutes to 24 hours after infection. Candida clearance was delayed in mice treated with cortisone. Thus, Monilia census at the termination of steroid treatment was commonly similar to that observed immediately after the initiation of infection. In no animal, however, was there evidence of progression of infection within the liver, spleen, lungs, or brain, and in each of these tissues the fungus was rapidly cleared as soon as cortisone treatment was stopped. These findings are shown in Figure 5 in which the effects of cortisone on liver and lung populations are summarized, the results being similar in the spleen and brain.

Candida census rose more than 2 standard deviations above mean values observed during the first

24 hours of infection in 2 of 40 hearts. In the other 38 mice, clearance was delayed during cortisone administration but no progression of infection was observed.

Studies on moribund animals dying within the first week of infection. The studies detailed above indicated that cortisone enhancement of moniliasis in mice was usually associated with increase in infection only in the kidneys. In an effort to see if fungus multiplication could be induced in other organs, mice were infected with 5×10^6 cells and were sacrificed for tissue population studies if they became moribund during the first 7 days of infection. Cortisone regimens in these studies consisted of 0.5 mg daily, starting two days prior to infection and continuing until sacrifice, or 2.5 mg the day prior to, the day of, and the day after infection.

The results of these studies are depicted in Figure 6 in which populations observed when mice were moribund are compared with populations found 4 hours after infection.

Kidney populations 4 hours after infection varied from 4.8×10^4 to 3.0×10^5 and rose to 7.84×10^6 to 1.7×10^8, the average increase being 500-fold.

Heart census ranged from 2.3×10^4 to 3×10^5 4 hours after infection and increased to 9×10^4 to 3×10^6 when mice were terminally ill. The mean increase was 17 times the 4 hour titer. Lung populations varied from 2.2×10^4 to 1.2×10^5 4 hours after inoculation. The numbers of Candida in the lungs of moribund mice ranged from 3.54×10^4 to 2.8×10^5, the average increase being only fourfold.

Liver census 4 hours after infection varied from 2×10^5 to 6×10^5. Populations in moribund mice had increased an average of 2.5 times and ranged from 8.4×10^4 to 2.16×10^6.

Brain census varied from 3.3×10^4 to 5.8×10^4 4 hours after infection and ranged from 3.1×10^4 to 8×10^5 when animals were terminally ill, an average rise of fivefold.

Thus, even in mice dying rapidly of overwhelming candidiasis, cortisone enhancement was limited almost exclusively to the kidneys. Liver, lung and brain populations showed only a minimal increase and heart census rose moderately. Whether the

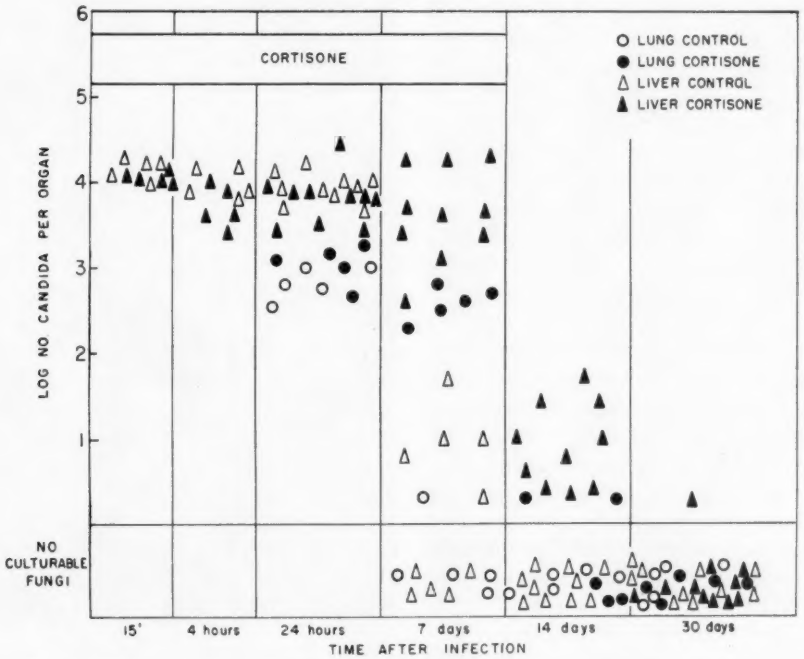

FIG. 5. EFFECT OF CORTISONE ON LIVER AND LUNG POPULATIONS IN MICE INFECTED I. V. WITH 10^5 CELLS OF *C. ALBICANS*. Cortisone was administered subcutaneously in dosage of 0.5 mg per day starting two days prior to infection.

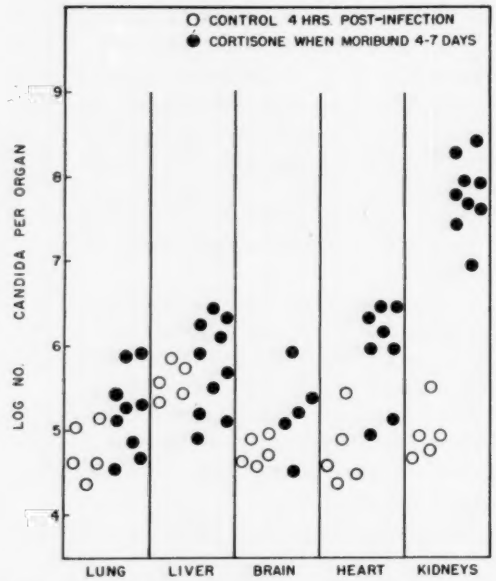

FIG. 6. TISSUE POPULATIONS IN CORTISONE-TREATED MORIBUND ANIMALS DYING DURING THE FIRST SEVEN DAYS OF INFECTION WITH 5×10^6 CELLS OF *C. ALBICANS*.

small increases in heart, lung, liver and brain census actually represented multiplication of the fungus within these tissues was uncertain, since moribund mice invariably demonstrated fungemia which ranged from 20 to 300 cells per ml of blood.

Effect of delaying cortisone treatment. The effects of cortisone were markedly reduced when treatment was delayed. When the hormone was administered prior to infection with 5×10^6 cells, 27 of 30 mice (90 per cent) died during the observation period. When treatment was begun the day after infection mortality was slightly diminished, 14 of 20 (70 per cent) animals dying within 30 days (see Figure 3). Delay in starting cortisone until 1 week after infection further reduced the mortality to 3 of 17 mice (17.2 per cent). This latter figure was not significantly different from the 11.7 per cent death rate observed in control mice infected with 5×10^6 cells.

An analysis of tissue populations confirmed the studies on mortality rates. When 0.5 mg cortisone was administered prior to infection with 10^5 to 5×10^6 cells, the average rise in renal census during the initial seven days of infection was 1,495-fold. Delay until the infection was established for 24 hours decreased the extent of enhancement so that kidney populations rose only an average of 386 times the mean census observed immediately after infection.

If cortisone was withheld until the infection was seven days old the effects of the steroid were reduced further, the mean increase in renal census being only 49-fold. Thus, the time of administration of cortisone appeared to be of major importance in the degree of augmentation of infection.

The effects of cortisone in mice challenged with small inocula of C. albicans. Infections due to *C. albicans* in man are probably initiated with small numbers of fungi. It seemed important, therefore, to determine whether cortisone could significantly increase tissue populations under experimental conditions in which the infecting inoculum was small.

Two experiments were performed in which mice were infected with 10^3 cells and were treated with 0.5 mg cortisone daily beginning two days prior

FIG. 7. BUDDING FORM OF *C. ALBICANS* SURROUNDED BY NEUTROPHILS IN A CORTICAL CAPILLARY OF THE KIDNEY OF A CORTISONE-TREATED MOUSE 6 HOURS AFTER I. V. INOCULATION WITH 10^7 ORGANISMS. (×2,100; PAS-hematoxylin.)

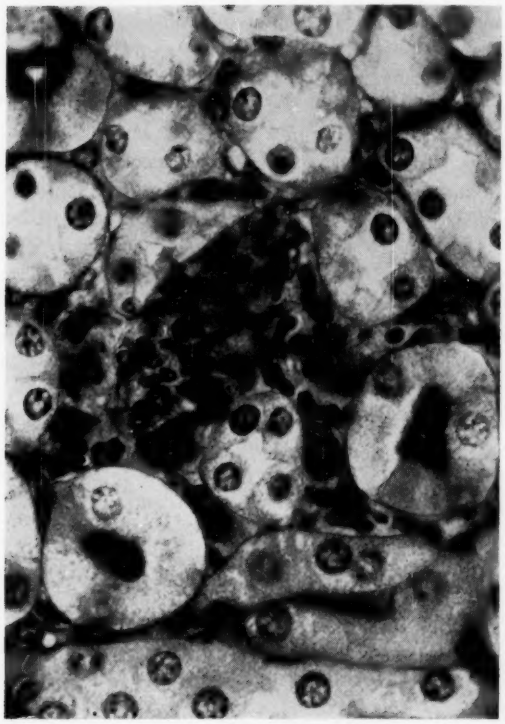

FIG. 8. INTERSTITIAL LESION (MICROABSCESS) IN THE CORTEX OF A KIDNEY OF A CORTISONE-TREATED MOUSE 24 HOURS AFTER I. V. INJECTION WITH 10^7 ORGANISMS. Proliferating fungi are surrounded by polymorphonuclear leukocytes. The inflammatory cells and fungi in the expanding lesion compress the surrounding tubules. (× 1,200; PAS-hematoxylin.)

to infection and continuing for nine days. Renal census was determined at the termination of cortisone treatment.

Kidney populations varied from 0 to 3×10^4 4 hours after infection. Seven days after infection renal census was less than 10^4 in 8 of 10 control animals and less than 10^3 in all 10. On the other hand kidney populations were greater than 10^3 in 7 of 11 cortisone-treated animals and were greater than 10^5 in 5 of these 7. There was then clear evidence that cortisone could cause marked increase in infection even if the inoculum was small.

Histological studies on normal mice. The initial lesions were similar in all tissues following intravenous inoculation. Fungal spores were trapped in the capillaries of the lungs, liver, spleen, heart, pancreas, adrenals, skeletal muscle and kidneys. Touch preparations showed that as early as 0.5 hour after injection, neutrophils, and macrophages had phagocytized some spores. Others were found free within the capillaries as late as 24 hours after inoculation.

Four hours after infection, budding and hyphal forms, often surrounded by neutrophils, had begun to appear within the vessels (Figure 7). As growth continued, the hyphae broke through the capillary wall into the interstitial tissues. Thereafter, necrosis occurred, and this was accompanied by a more intense cellular response, which reached a peak in from 24 to 30 hours with formation of frank abscesses (Figure 8). Micro-abscesses occurred in the liver, kidney, pancreas, heart and skeletal muscle, but only rarely in the lungs, and never in the spleen or adrenals.

In all organs except the kidneys, the lesions invariably regressed. By the third day, they were

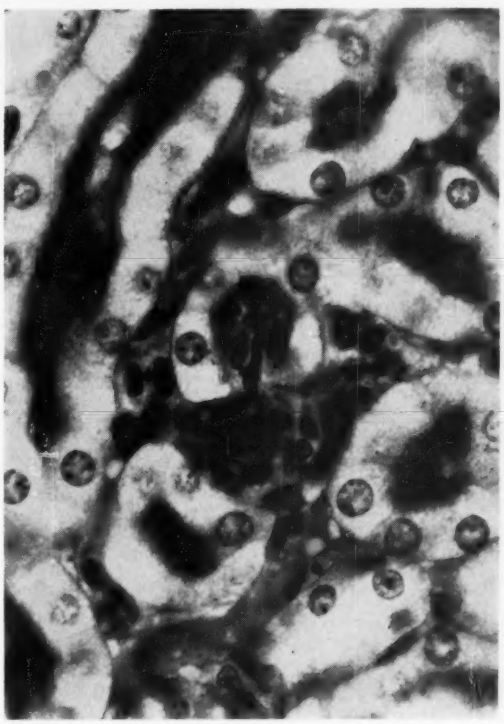

FIG. 9. A GROWING HYPHAL FORM OF *C. ALBICANS* RUPTURING INTO THE LUMEN OF A PROXIMAL CONVOLUTED TUBULE IN THE KIDNEY OF A CORTISONE-TREATED MOUSE 24 HOURS AFTER INOCULATION WITH 10^7 ORGANISMS. (× 1,200; PAS-hematoxylin.)

generally inapparent or marked by small accumulations of histiocytes, in which fungi were seldom seen.

In the kidneys the interstitial lesions also commonly regressed, but progression of the lesions was occasionally observed. Such progression was associated with rupture by the fungus through the walls of both cortical and medullary tubules or glomerular tufts into the lumina of the nephrons (Figure 9).

Once within the lumen of the tubule the fungi multiplied rapidly and grew proximally and distally (Figure 10) forming long hyphal elements. Growth in this apparently protected site was not associated with the inflammatory response which was so prominent in the interstitium. Polymorphonuclear leukocytes were carried into the tubules with the fungi. These became pyknotic and karyorhectic. No further polymorphonuclear infiltration occurred during this stage in the development of the lesion, despite the extensive intraluminal multiplication of Monilia.

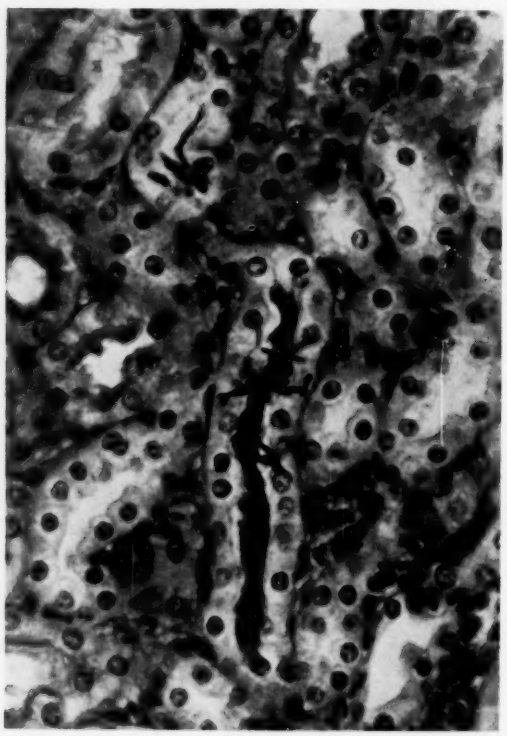

FIG. 10. RAPID INTRATUBULAR GROWTH OF *C. ALBICANS* WITHIN THE LUMEN OF A DISTAL CONVOLUTED TUBULE OF THE KIDNEY OF A CORTISONE-TREATED MOUSE 24 HOURS AFTER I. V. INOCULATION WITH 10^7 ORGANISMS. Growth within the tubule is unaccompanied by polymorphonuclear leukocytic response. (× 675; PAS-hematoxylin.)

Mycelial casts with or without necrotic polymorphonuclear leukocytes were washed into the medulla and were caught in the loops of Henle or collecting tubules. There the fungi continued to grow rapidly and, as in the lumina of the cortical tubules, at first without cellular response.

Within 24 hours of the time of inoculation, however, many of the large hyphae growing within the tubules had once more broken out into the interstitium and adjacent nephrons. The reappearance of the fungus in the interstitium was associated with an intense inflammatory response. This time the larger hyphal elements formed the foci of much more intense and destructive cortical and medullary abscesses than had been produced when the growing spores first penetrated the capillary wall.

In the occasional control animal in which this type of progressive lesion developed, but which nevertheless survived beyond the third day after infection, the large cortical abscesses regressed. The lesions became localized by the accumulation of many macrophages and proliferating fibrous tissue at their peripheries. The hyphae and spores were ingested by the macrophages, became fragmented, lost their staining characteristics, and gradually disappeared. By 14 days after inoculation, such lesions were only identified by cellular scars in the cortex.

Pathogenesis of the lesions in cortisone-treated mice. Early lesions caused by the fungus were similar to those described in control animals. By the methods used, polymorphonuclear infiltration and phagocytosis appeared to occur with equal rapidity in cortisone-treated and control mice. The subsequent lesions observed 4 to 48 hours after infection were more florid and more numerous, however, in all organs examined in the mice treated with cortisone. The interstitial abscesses in the liver, kidney, pancreas, heart, and skeletal muscle had by 24 to 48 hours reached greater dimensions. They contained more neutrophils and fungi, either viable or disintegrating, and involved the destruction of more parenchymal cells.

The renal lesions invariably progressed. Rupture of the fungus into the tubular lumen occurred far more extensively than in control animals, and

FIG. 11. A KNOT OF PROLIFERATING HYPHAE BREAKING FROM THE LUMEN OF A COLLECTING TUBULE INTO THE INTERSTITIAL TISSUE OF THE RENAL MEDULLA OF A CORTISONE-TREATED MOUSE 24 HOURS AFTER I. V. INOCULATION WITH 10^7 ORGANISMS. (× 1,200; PAS-hematoxylin.)

was followed by rapid intratubular growth unassociated with intratubular inflammatory response. Within 24 hours, the fungi growing within tubules had again penetrated the tubular walls to break back into the interstitium. Polymorphonuclear response to this recurrent interstitial invasion was intense with the production of large destructive cortical and medullary abscesses. The burgeoning mycelial knot that developed from the rapid intratubular growth of the fungi and then broke out into the interstitium appeared much less susceptible to phagocytosis than did the yeast cells and the isolated pseudomycelial growths that had broken into the tubules (Figure 11).

In the kidneys of cortisone-treated mice at 24 hours, then, there was a large number of cortical and medullary abscesses, as well as a smaller number of blood-borne interstitial lesions and proliferating intratubular casts in the medulla.

By the third day, and thereafter until the seventh day, retrograde and lateral spread of these lesions led to the production of subcapsular granulomas and infarct-like lesions (Figure 12). Large masses of mycelia grew from the papillae into the calyces. Parenchymal abscesses took the form characteristic of mycetomal lesions with a central mycelial knot surrounded by a wide zone of polymorphonuclear leukocytes and a periphery of macrophages, lymphocytes, fibroblasts, and occasional giant cells.

From the third to the seventh day new lesions appeared in the kidneys of the cortisone-treated mice but not in those of the control mice. Discovery of eroded walls of veins at the periphery of abscesses in steroid-treated animals suggested

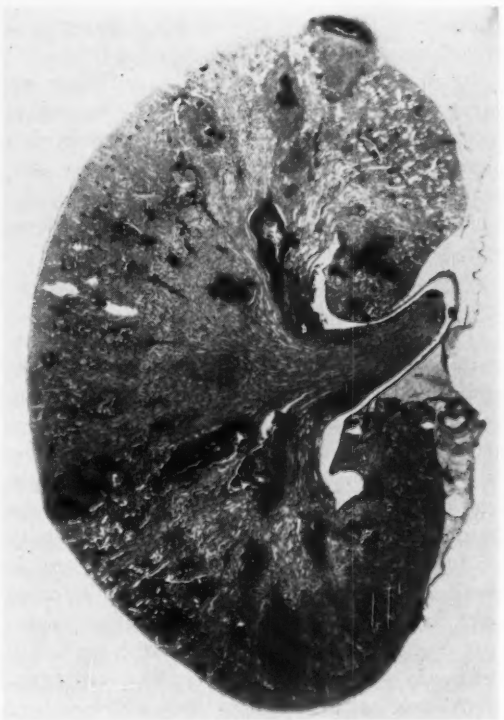

FIG. 12. MULTIPLE LESIONS IN THE KIDNEY OF A CORTISONE-TREATED MOUSE 7 DAYS AFTER I. V. INOCULATION WITH 10^6 ORGANISMS. Large mycelial masses (black areas) are surrounded by an inner zone of polymorphonuclear leukocytes and by an outer zone of macrophages and fibroblasts. Wedge-shaped, infarct-like lesions are distributed throughout the cortex. Fungi grow from the parenchyma of the medulla into the lumen of the pelvis. (× 12; PAS-hematoxylin.)

a means by which the continued dissemination of the infection took place.

In the mice that survived after the seven to nine day period of cortisone administration, there was gradual resolution of the lesions and replacement of destroyed renal parenchyma by scar tissue.

The only other tissue besides the kidney in which extensive lesions were observed was heart muscle. Here the lesions in cortisone-treated mice usually regressed in a similar fashion to that in control mice. However, in some of the steroid-treated mice, which were sacrificed when moribund five to seven days after infection, myocardial abscesses which contained many fungi had developed, and caused destruction of much myocardium. Simultaneous tissue census studies on such animals (see above) demonstrated that heart populations rose only 17-fold as compared to a rise of approximately 500-fold in renal titers.

In all other tissues, the lesions uniformly regressed in a fashion similar to that observed in control animals, although healing was somewhat slower. By the third day the lesions were generally unapparent or marked by small accumulations of histiocytes in which fungi were seldom seen.

DISCUSSION

These studies suggest that the increased mortality in cortisone-treated mice infected with this strain of *C. albicans* was related specifically to increased multiplication of the fungus in the kidney, the organ which is the site of maximum infection in normal animals. In extra-renal tissues, cortisone treatment commonly delayed fungus clearance but only rarely (in the heart) was there evidence of increase in Candida populations. Even in moribund animals dying rapidly after infection with large numbers of *C. albicans*, fungus census was only moderately increased in the heart, and minimally elevated in the liver, lung and brain. Such elevations in populations may well have been related to the marked fungemia observed in these animals.

The kidney is the site of maximum multiplication in numerous other experimental infections, including those due to staphylococci (19), *Mycobacterium fortuitum* (20), and *Corynebacterium renale* (21). The mechanisms underlying the renal susceptibility to infection have not been delineated. The histological studies detailed above offer a plausible explanation for the localization of progressive infection to the kidneys in both normal and cortisone-treated mice infected with *C. albicans*.

The initial cellular response to invasion of the kidneys by Candida was polymorphonuclear and occured both within vessels and in the interstitium. Within 24 hours after infection, fungi broke from the interstitium apparently by mechanical pressure into the cortical and medullary renal tubules. Blyth (22), and Evans and Winner (23), in studies on normal animals, have also observed the growth of pseudomycelia into tubular lumina. Once within the tubular lumen, the fungus appeared protected from host defenses and grew rapidly forming long hyphal elements. Cellular defenses of the host did not appear to be effective until these hyphae again broke through the tubule wall back into the interstitial tissues.

The extent of intratubular growth appeared to determine the outcome of infection. When normal mice were given large inocula, intratubular multiplication and subsequent abscess formation were commonly limited, and the animal usually survived. In cortisone-treated animals injected with such inocula, intratubular growth was massive. This was followed by marked abscess formation and death.

In contrast to the events described in the kidney, histological study of other tissues revealed regression of lesions 24 to 72 hours after infection in control and cortisone-treated mice. Extensive extra-renal lesions were found only in the hearts of moribund animals dying four to seven days after infection with 5×10^6 cells.

The apparent ability of *C. albicans* to evade host defenses by intratubular residence would appear to be analogous to the protection afforded other microbes by an intracellular habitat. Thus, for example, Rogers showed that staphylococci incorporated into polymorphonuclear leukocytes were far better able to withstand passage through reticuloendothelial organs than were staphylococci which remained extracellular (24).

The hypothesis offered here that the renal localization of Candida infections in normal and cortisone-treated animals is related to the protection offered the fungus by intratubular residence has not been invoked before to explain the suscep-

tibility of the kidney to experimental or clinical infections. Whether intratubular growth may also account for renal localization of infections other than moniliasis is not known. The evidence presently available, however, suggests that different mechanisms may be of far greater importance in certain infections. Studies by Freedman and Beeson (25) have demonstrated that the renal medulla is more susceptible to *Escherichia coli* infections than is the renal cortex. Recent studies by Beeson and Rowley (26) suggest that the medullary susceptibility may be related to local inactivation of serum bactericidins by ammonia. It seems unlikely that such a mechanism is of major significance in moniliasis. The localization of fungal cells can be determined readily by histological studies of the kidney. There is no medullary predilection in Candida infections. Studies in our laboratory, which will be described in detail subsequently, demonstrate no inhibitory effect of mouse serum or plasma on *C. albicans*.

Although serial determinations of Monilia populations in several extra-renal tissues including lungs, liver, spleen, heart and brain demonstrated no increase in the number of recoverable fungi in mice treated with cortisone over the initial 24 hours of infection as compared with control animals, simultaneous histological studies showed clear evidence of increased growth of the fungus and augmented infiltration by neutrophils during this period. The enhanced infiltration by leukocytes appeared to be an appropriate reaction to the increased multiplication of the fungus. The early events in the kidney were similar to those observed in other tissues. Increased Candida multiplication within vessels and in the interstitial tissues was associated with a more prominent cellular infiltrate.

Failure of the bacteriological studies to demonstrate the increased growth observed microscopically suggests the possibility that the increased growth of Candida was balanced by increased destruction of the fungus. It is also possible that small increases in the number of Candida cells may not have been detectable by the quantitative enumeration techniques employed. Progressive infection failed to develop in extra-renal organs despite the early generalized increase in multiplication of fungi under the influence of cortisone and it seems that this may well be related to the inability of the fungus to find a site for protected growth within these tissues.

The time of administration of cortisone appears to determine the degree of enhancement of experimental Candida infections. Delay in steroid administration until 24 hours after infection with 5×10^6 cells decreased the mortality from 90 to 70 per cent. Further delay in initiation of cortisone treatment until seven days after infection markedly reduced the effects of the steroid so that the mortality was not significantly different from that noted in control animals, and only a modest increase in renal Candida census was observed. These studies are then in agreement with experiments on other microorganisms which indicate that the effects of steroids are reduced markedly if treatment is withheld until host defenses are established (27, 28).

Under the conditions of the present experiments, Candida infections were clearly enhanced to a far greater degree than infections due to *H. capsulatum* or *C. neoformans*. These findings do not necessarily contradict previous work demonstrating enhancement of experimental histoplasmosis or cryptococcosis by adrenal steroids (2–6). In the studies herein reported, experiments on *H. capsulatum* and *C. neoformans* were performed on NIH mice. It has been emphasized that the strain of animal used may influence to a great extent the results of studies on the effects of cortisone on a particular infection (29). It seems quite possible that the use of certain mouse strains would have been associated with augmentation not only of candidiasis but also of histoplasmosis and cryptococcosis. Indeed, limited experiments in this laboratory with CFW strain mice have demonstrated significant enhancement of cryptococcosis under the influence of cortisone. Nevertheless, the demonstration of differences among the three fungi in the degree of enhancement of infection by steroids under certain experimental conditions suggests that similar alterations induced by cortisone in the host may clearly favor the establishment of microbial parasitism by some mycotic species rather than by others. It is conceivable that these studies may offer a partial explanation for the well documented observation that the administration of adrenal hormones to man is associated with the appearance of disseminated moniliasis far more often than histoplasmosis or cryptococcosis

despite the frequent presence of the latter two fungi in the exogenous and, in the case of *C. neoformans*, in the endogenous environment of man.

It is important to emphasize that marked increase in renal Candida populations were noted in most cortisone-treated animals even when the infecting inoculum was small. One of the difficulties in applying experimental knowledge to human infection is the necessity for using very large inocula in the experimental study of microorganisms of low pathogenicity such as Monilia. Human infections on the other hand are probably initiated with small numbers of microorganisms. Since cortisone produces marked enhancement of Candida infections initiated with small inocula, and since the kidney is the organ of maximum infection in both man and mouse (30), there is a considerable possibility that the experimental findings herein described may contribute to an understanding of some cases of disseminated moniliasis in man.

SUMMARY

Experimental Monilial infections in mice were enhanced to a far greater degree by cortisone than were infections due to *Histoplasma capsulatum* or *Cryptococcus neoformans*.

The kidney was demonstrated to be the only tissue in which progressive infection occurred in normal mice following intravenous injection of *Candida albicans*. The administration of cortisone was associated with striking enhancement of the renal infection. Tissue Candida clearance was delayed in extra-renal sites during cortisone treatment but increase in Monilial census was observed only rarely (in the heart).

Histological studies suggested that the renal localization of infection in normal and cortisone-treated mice was related to rupture of the fungus into tubular lumens. Once within the tubule the fungus appeared protected from host defenses and grew in an uninhibited fashion. Extensive intratubular residence was uncommon in normal mice injected with 10^5 to 5×10^6 cells but occurred uniformly in cortisone-treated animals.

Enhancement of renal infection was also observed in most cortisone-treated animals injected with small inocula. The maximum effect of cortisone was produced when animals were given treatment from two days prior to infection. Delay in the initiation of cortisone treatment until the day after infection reduced the effects of the hormone. When cortisone was administered beginning seven days after infection no significant enhancement was observed.

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RELATIONSHIP BETWEEN $Rh_0(D)$ GENOTYPE AND QUANTITY OF I^{131} ANTI-$Rh_0(D)$ BOUND TO RED CELLS *

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The antigens on the stroma of the red cell represent immunochemically specific molecules whose synthesis is genetically determined (1). The genetic control of the blood group antigens in man was recognized (2) within a decade after their discovery by Landsteiner. If the one-gene-one product hypothesis applies to the blood group antigens, the red cell antigen content should be related quantitatively to the genotype. The results obtained with techniques based on agglutination of red cells suggest that a dosage effect exists for many of the blood group systems (3). Some cells agglutinate more intensely than others with a given antibody indicating that these cells possess more antigen. Under these circumstances, it has been inferred that the red cells reacting more intensely with the antibody are genetically homozygous for the antigen whereas the weakly reacting cells are heterozygous.

Except for R^2R^2 (cDE/cDE) and $R^{ox}R^{ox}$ (-D-/-D-) red cells which react more strongly with anti-$Rh_0(D)$, no clear-cut dosage effect has been demonstrated for the $Rh_0(D)$ antigen (1). Grubb (4) using an antiglobulin absorption and inhibition technique was able to estimate the $Rh_0(D)$ receptor sites of the red cell but was unable to differentiate the heterozygous from the homozygous $Rh_0(D)$ red cell. Boursnell, Coombs and Rizk (5) have applied serum containing I^{131}-labeled anti-$Rh_0(D)$ to this problem. They did not derive any conclusions with respect to the $Rh_0(D)$ genotype because of the high nonspecific binding of I^{131}-labeled proteins to the red cells. Wiener and Gordon (6) have applied a quantitative antiglobulin test to this problem, but do not indicate that they were able to determine the $Rh_0(D)$ genotype with their technique.

The availability of a relatively specific I^{131} incomplete anti-$Rh_0(D)$ (7) provided the opportunity to study the relationship between the $Rh_0(D)$ genotype and the $Rh_0(D)$ antigen content of the red cell. The experimental design adopted for this study was to determine the $Rh_0(D)$ antigen content of different red cells obtained from the $Rh_0(D)$ positive donor population entering the Central Blood Bank of Pittsburgh. The frequency distribution of the red cell $Rh_0(D)$ content in this population of red cells was determined graphically, and the heterozygous state in this distribution was identified by studying genetically defined $Rh_0(D)$ heterozygotes obtained from family studies. This plan of study was used because there is no conventional technique [absence of both dosage effect and anti-rh (d) serum] for positively differentiating the $Rh_0(D)$ heterozygous red cell from the homozygous red cell. A preliminary report has been published (8, 9).

MATERIALS AND METHODS

Except for the procedures described below, the methods presented in a previous paper (7) were used for iodination,[1] determination of radioactivity, stroma preparation and reaction of the red cells with the I^{131} anti-$Rh_0(D)$.

Fractionation of anti-$Rh_0(D)$ serum. High titered anti-$Rh_0(D)$ serum was obtained from a 30 year old white housewife who had been immunized to the $Rh_0(D)$ antigen by multiple pregnancies. The pertinent details of her history and immunization have been presented (7).

The γ-globulins were isolated by the method of Nichol and Deutsch (10), in place of the modified Cohn method 10 procedure used previously (7). The titer of the anti-$Rh_0(D)$ serum before fractionation was 2,048 and the titer of the γ-globulin fraction was 2,048. The anti-

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[1] Carrier-free I^{131} was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

TABLE I

Properties of the I^{131} anti-$Rh_0(D)$ γ-globulin fraction

Per cent of I^{131} bound	13.70
I (moles/mole globulin)*	0.98
I^{131} ($\mu c/\mu g$ N)†	0.15
Per cent of I^{131} precipitable with trichloroacetic acid	97.7
Titer before iodination	2,048
Titer after iodination	512
Nitrogen (mg/ml)	2.54

* Calculated using a molecular weight of 160,000 for the total fraction iodinated.

† Activity at the time of iodination.

$Rh_0(D)$ titers were determined with the antiglobulin reaction using pooled $Rh_0(D)$ positive red cells, volumetric dilutions of the anti-$Rh_0(D)$ preparation, and commercial antiglobulin reagent (11). The γ-globulin fraction contained 8.04 per cent of the total serum nitrogen as determined by the micro-Kjeldahl method (12). The properties of the I^{131} iso-antibody preparation are listed in Table I.

Elution of I^{131} anti-$Rh_0(D)$ from $Rh_0(D)$ stroma. A modification of the elution technique previously reported (7) was used for these studies. The pooled lyophilized $Rh_0(D)$ stroma was sensitized with the I^{131} anti-$Rh_0(D)$ γ-globulin at 37° C for 1 hour. To 10 g of stroma was added 100 ml of the I^{131}-globulin which contained 0.10 to 0.16 mg N per ml dissolved in pH 6.5, 0.15 M phosphate buffer with 0.03 per cent human γ-globulin carrier.[2]

The stroma, which had been sensitized in plastic tubes with a capacity of 30 ml was washed 4 times with the phosphate buffer by centrifugation (Spinco model L ultracentrifuge, 15 minutes at 20,000 rpm, maximum G 46,900).

The I^{131} anti-$Rh_0(D)$ was eluted from the sensitized stroma by adjusting the pH, temperature, and duration of elution. A preliminary eluate which contained mostly nonspecific I^{131}-globulins was obtained by eluting the sensitized stroma for 5 minutes at 56° C with pH 6.0, 0.15 M phosphate buffer containing 3 per cent γ-globulin carrier. This eluate was recovered by 15 minutes' centrifugation at room temperature (20,000 rpm, maximum G 46,900) and was discarded. The I^{131} anti-$Rh_0(D)$ was obtained by a second elution at 56° C for 30 minutes with pH 5.8, 0.15 M phosphate buffer containing 0.1 per cent γ-globulin carrier. The second eluate which contained biologically active I^{131} anti-$Rh_0(D)$ was separated from the stroma by centrifugation (15 minutes at 20,000 rpm, maximum G 46,900) in a Spinco model L ultracentrifuge using a rotor preheated to 60° C. The tubes containing the centrifuged stroma were placed in a 56° C water bath immediately after centrifugation and the supernates containing the eluted I^{131} anti-$Rh_0(D)$ were recovered by aspiration. The eluate obtained in this fashion was dialyzed overnight at 4° C against pH 6.5, 0.15 M phosphate buffer containing 1:10,000 merthiolate and 0.1 per cent γ-globulin carrier to readjust the pH of the eluates.

The eluates used in these studies were obtained from a pool of $Rh_0(D)$ positive red cell stroma derived from 16 units[3] of outdated whole blood. The Rh phenotypes of the units making up this pool were as follows: 7 units of R^1r (CDe/cde) or R^1R^0 (CDe/cDe); 4 units of R^2r (cDE/cde) or R^2R^0 (cDE/cDe); 3 units of R^1R^1 (CDe/CDe) or R^1r' (CDe/Cde) and 1 unit each of R^2R^2 (cDE/cDE) or R^2r'' (cDE/cdE), and R^2r (cDE/cde) or R^2R^0 (cDE/cDe). A total of 107 g of red cell stroma was obtained from these 16 units of whole blood.

Three different eluates were derived from this one pool of stroma. The properties of these eluates are listed in Table II. Eluate P-1 was obtained after reacting the globulin containing I^{131} anti-$Rh_0(D)$ (72 mg N) with 90 g of the stroma. Eluate P-2 was obtained by pooling 112 ml of P-1 and 40 ml of the supernates recovered from the studies in which $Rh_0(D)$ negative red cells had been reacted with P-1. Eluate P-3 was made up by pooling the $Rh_0(D)$ negative supernates derived from P-1 and P-2. This device of saving and pooling the supernates containing I^{131} anti-$Rh_0(D)$ from $Rh_0(D)$ negative red cells was used to achieve maximum utilization of the I^{131} anti-$Rh_0(D)$. The immunochemical activity of all three eluates was directly proportional to their total nitrogen content and was unaffected by previous reaction (absorption) with $Rh_0(D)$ negative red cells. The specific antibody content of each of the three eluates was determined by reacting the supernate with an excess of $Rh_0(D)$ positive red cells. The total antibody content was obtained by adding the antibody found in the supernate to that taken up by the original $Rh_0(D)$ positive red cell. These values are found in Table III.

The I^{131} γ-globulin fraction and the I^{131} anti-$Rh_0(D)$ eluate were studied by zone electrophoresis on potato starch with 0.1 ionic strength, pH 8.6 barbital buffer (13). Both the γ-globulin and the eluate preparations revealed a single symmetrical peak of radioactivity at the origin and a small albumin peak which had migrated away from the origin toward the anode. The distribution of protein in the starch block was associated with the I^{131} radio-

TABLE II

Properties of I^{131} anti-$Rh_0(D)$ eluates

Preparation	TCA-precipitable I^{131}*	Titer	Nitrogen
			μg/ml
P-1	87.0	16	3.96
P-2	86.1	16	3.83
P-3	89.2	4	2.92

* Trichloroacetic acid, 10 per cent.

[2] Obtained from Cutter Laboratories, Berkeley, Calif. The γ-globulin fraction was free of anti-$Rh_0(D)$ when tested with the antiglobulin reaction.

[3] These were conventional blood bank units consisting of 480 ml of whole blood and 120 ml acid citrate dextrose solution.

TABLE III

Antibody content of I^{131} anti-$Rh_0(D)$ eluates

Preparation	Nitrogen	No. of determinations	Antibody nitrogen*	Antibody nitrogen
	μg/ml		μg/ml	%
P-1	3.96	32†	0.127 ± 0.009	3.21
P-2	3.87	10	0.110 ± 0.015	2.85
P-3	2.92	4	0.0833 ± 0.0067	2.86

* Standard error of the mean.

† Seven of these determinations were based on reacting the supernates with the red cell suspension that was used for the original reaction. All the other values were obtained by reacting the supernates with a different $Rh_0(D)$ positive red cell sample.

activity. The antibody was localized on the cathode side of the protein peak at the origin.

Source of red blood cells. Red cells were obtained from the Caucasian donor population entering the Central Blood Bank of Pittsburgh and from the Blood Bank staff. The red cell samples were not accepted randomly from the donor population. $Rh_0(D)$ positive red cells were selected to conserve the supply of I^{131} anti-$Rh_0(D)$. Only 1 or 2 $Rh_0(D)$ negative samples were accepted each day to provide the necessary controls for the study. $Rh_0(D)$ negative cells or donors who did not know their Rh phenotype were rejected from the study. The red cell samples were obtained from venous blood anticoagulated with heparin. Some of the red cells were reacted with the I^{131} anti-$Rh_0(D)$ on the day they were collected and others were reacted after periods of 1 to 6 days of storage in Alsever's solution at 4° C. The cells were typed for the ABO and Rh antigens using commercial antisera [4] and the methods recommended by the manufacturer. The rh'(C) positive red cells included the $rh^w(C^w)$ antigen since a specific anti-$rh^w(C^w)$ was not used to test for this antigen.

TABLE IV

Experimental errors

Procedure	Degrees of freedom	Variance, 10^{-4} μg N/0.01 ml centrifuged RBC
Daily determinations on the same sample of stored red cells*	9	1.63
Daily determinations on the same unstored red cell sample*	5	2.28
Duplicate determinations after storage on different red cell samples	7	3.58
Duplicate determinations on the same cell suspension	11	0.678

* These data are derived from the results presented in Table II.

[4] I am grateful to the Ortho Research Foundation, Raritan, N. J. for generous supplies of anti-hr'(c).

A total of 354 individual determinations was carried out with the three eluates obtained from the pooled red cell stroma. The results to be discussed in this paper are based on the following studies: 199 different Caucasian red cell samples; 21 family study determinations; and 23 control determinations on $Rh_0(D)$ negative red cell samples. Eighty of the 354 determinations represented duplicate determinations, supernate studies and various miscellaneous studies. Excluded from this report are the studies carried out on 31 different samples of Negro red cells.

A series of five families with eight children in whom a documented history of Rh incompatible pregnancy was present was used for the source of $Rh_0(D)$ heterozygous cells.[5]

Calculations and presentation of results. The red cells were exposed to the I^{131} anti-$Rh_0(D)$ at 37° C for 1 hour. Cell concentration was determined by the microhematocrit method (14, 15). The results are expressed as micrograms of antibody nitrogen bound to 0.01 ml centrifuged red cells as defined by 5 minutes' centrifugation at maximum G 12,000 in an International micro-hematocrit centrifuge. The I^{131} bound to the red cells was converted to antibody nitrogen using the I^{131} to nitrogen ratio of the total γ-globulin fraction before elution. The nitrogen of this fraction could be determined with the micro-Kjeldahl procedure (12) but the nitrogen content of the eluates containing I^{131} anti-$Rh_0(D)$ was insufficient for direct determination. Justification for the conversion of I^{131} to nitrogen is based on the finding that the iodine to nitrogen ratio of specific antibody is the same as that of the total γ-globulin fraction (16). The I^{131} to nitrogen ratio has also been used to determine ultra-microgram quantities of I^{131} diphtheria toxin nitrogen (17). In this study the nitrogen values which were based on I^{131} radioactivity were confirmed independently by toxicity studies.

RESULTS

Effect of storage and over-all precision. The over-all reproducibility of the technique including the effects of storage was evaluated by storing a

[5] I am greatly indebted to Drs. Paul Gaffney and William Chapman of Pittsburgh, Pa., who made their patient material available for these studies.

large volume of red cells in Alsever's solution at 4° C. On subsequent days an aliquot of the stored cells was reconstituted and reacted with the I^{131} anti-$Rh_0(D)$. A fresh specimen of cells obtained by venipuncture from the donor of the stored cells was reacted along with the stored cells with the I^{131} anti-$Rh_0(D)$. The pooled variance for repeated determinations on the stored cells over a period of 11 days was 1.63×10^{-4} μg N per 0.01 ml RBC (Table IV) and the mean and standard error of the mean was $1.85 \pm 0.13 \times 10^{-2}$ μg N per 0.01 ml RBC [6] (Table V). The corresponding values for the determinations carried out on fresh unstored red cells were 2.28×10^{-4} (Table IV) and 1.89 ± 0.15 (Table V). There was no significant difference in the mean values between the fresh and stored red cell samples ($t = 0.18$, $n = 14$).

The over-all precision was evaluated in another way by repeating the determination on the same red cell suspension after a period of one to four days of storage at 4° C. The pooled variance for seven such different cells was 3.58×10^{-4} μg N per 0.01 ml RBC (Table IV).

The pooled variance for duplicate determinations on the same cell suspension for 11 different red cell samples was 0.678×10^{-4} μg N per 0.01 ml RBC (Table IV). This statistic excludes the errors that occur in preparing a cell suspension with a known hematocrit. It evaluates only the errors associated with the procedure of reacting the cells with the labeled iso-antibody—pipetting errors, loss of red cells both physically and by hemolysis during the procedure, and errors in the determination of radioactivity.

TABLE V

Effect of storage on quantity of antibody bound to the same sample of red cells

Age	Nitrogen bound, 10^{-2} μg/0.01 ml centrifuged RBC	
	Stored red cells	Fresh red cells
days		
0	1.79*	
1	1.75*	1.97*
2	1.85	1.79
3	1.97	1.89
4	2.08	
6	1.73	1.67
8	2.04	2.07
11	1.76	
Mean	1.85 ± 0.13	1.89 ± 0.15

* These values represent the average of duplicate determinations. The red cells used in this study were all obtained from one individual whose probable genotype was R^1r' (CDe/Cde).

[6] Mean and standard error of the mean are used throughout this paper.

TABLE VI

Number of red cells in 0.01 ml volume of centrifuged cells

Age	No. of samples counted	Mean number of cells $\times 10^7$ *
days		
0	5	9.37 ± 1.15†
1	15	8.44 ± 1.05
2	12	8.85 ± 1.93
3	8	9.07 ± 0.79
4	13	8.52 ± 1.63
5	8	9.10 ± 1.46
6	10	9.73 ± 2.04
7	4	8.75 ± 1.03
8	4	9.17 ± 1.14
9	6	10.13 ± 1.17

* The determinations were carried out with the Coulter electronic cell counter. The volume of centrifuged cells was defined by 5 minutes' centrifugation at maximum G 12,000.

† Standard error of the mean.

Relationship of hematocrit and cell count. The number of red cells in some of the cell suspensions was counted directly [7] by means of the Coulter electronic counter (18, 19). The cells had *in vitro* ages that ranged from 0 to 11 days. The mean number of red cells in a 0.01 ml volume of RBC, (defined by 5 minutes' centrifugation at maximum G 12,000) was $9.01 \pm 1.48 \times 10^7$ cells. The red cell count was directly proportional to the hematocrit even though there was considerable scatter in the data. The number of red cells was independent of the *in vitro* age of the red cell suspension (Table VI) indicating that the scatter in the data was not related to osmotic volume changes induced by the conditions of storage.

Nature and quantity of I^{131} bound to $Rh_0(D)$ negative red cells. The mean quantity of nitrogen bound to 23 samples of $Rh_0(D)$ negative red cells was $0.150 \pm 0.037 \times 10^{-2}$ μg nitrogen per 0.01 ml RBC. The quantity of nitrogen was independent of both the Rh or the ABO phenotype of both $Rh_0(D)$ and D^u negative cells, and represented from 3 to 7 per cent of the I^{131} bound to $Rh_0(D)$

[7] The interest and generous cooperation of Dr. R. C. Hamilton and of Sister Marietta (Registered Technologist, St. Francis Hospital, Pittsburgh, Pa.) in carrying out these determinations is gratefully acknowledged.

TABLE VII

Nitrogen bound to $Rh_0(D)$ negative red cells

Phenotype	No. of samples	10^{-2} μg Nitrogen bound to 0.01 ml centrifuged RBC
Distribution of samples by Rh phenotype		
rr (cde/cde)	16	0.160 ± 0.040*
r'r (Cde/cde)	3	0.130 ± 0.030
r''r (cdE/cde)	4	0.128 ± 0.015
Distribution of samples by ABO phenotype		
A	4	0.131 ± 0.034
B	3	0.126 ± 0.021
O	16	0.160 ± 0.038

* Standard error of the mean. The mean value for the 23 $Rh_0(D)$ negative red cells was 0.150 ± 0.037 × 10^{-2} μg N per 0.01 ml packed red cells.

positive cells. These results are tabulated in Table VII.

Criteria employed to determine antibody excess. The presence of antibody excess was ascertained by reacting $Rh_0(D)$ positive red cell supernates with unsensitized $Rh_0(D)$ positive red cells. The supernates were tested with either the same $Rh_0(D)$ positive cell or with an $Rh_0(D)$ positive cell obtained from a different individual (different Rh phenotype). All the tests on the supernates were carried out in the presence of a marked excess of antigen (large excess of red cells) so that an estimate of the total antibody content could be obtained.

Excess I^{131} anti-$Rh_0(D)$ could be demonstrated in all the supernates tested. The total antibody recovered by adding the antibody found in the supernate to that removed by the original $Rh_0(D)$ positive cell in six determinations was 0.126 ± 0.016 × 10^{-2} μg of antibody nitrogen per ml of I^{131} anti-$Rh_0(D)$. When the supernate was tested with a different $Rh_0(D)$ positive cell (26 determinations), the total antibody content was 0.127 ± 0.008 × 10^{-2} μg of antibody nitrogen per ml. These results indicate that the estimation of the total antibody content was independent of the type of $Rh_0(D)$ positive cell that was used to test the supernate. The value obtained using the same $Rh_0(D)$ positive red cell did not differ significantly from that found using a different positive red cell (Table VIII).

These data are plotted in Figure 1. The antibody nitrogen bound to the original red cell is plotted on the abscissa and the antibody nitrogen found in the supernate with the use of either the same cell or a different $Rh_0(D)$ positive red cell is shown on the ordinate. The quantity of antibody nitrogen remaining in the supernate was inversely proportional to the quantity of antibody removed by the original cell.

The degree of saturation of the available $Rh_0(D)$ sites on the red cell was also evaluated by re-reacting the washed I^{131} sensitized red cells with an additional quantity of I^{131} anti-$Rh_0(D)$. The increase in I^{131} anti-$Rh_0(D)$ bound to previously sensitized red cells after a second incubation of 60

TABLE VIII

Antibody excess as shown by free antibody in the supernate *

Phenotype original red cell	Antibody N bound to original red cell (A)	Phenotype red cell reacted with supernate	Antibody N found in supernate (B)	Total antibody N recovered (A+B)
R^1r or R^1R^0	4.40	Same cell	6.42	10.8
R^1r or R^1R^0	5.25	Same cell	5.88	11.1
R^1r or R^1R^0	8.84	Same cell	4.99	13.8
R^1r or R^1R^0	5.53	Same cell	6.04	11.6
R^1R^1 or R^1r'	8.59	Same cell	5.31	13.9
			Mean	12.2
R^1r or R^1R^0	5.34	R^1R or R^1r'	6.76	12.1
R^1r or R^1R^0	6.38	R^1R or R^1r'	5.80	12.2
R^1R^2 or $R'r''$	9.12	R^1R^1 or R^1r'	4.15	13.3
R^2r or R^2R^0	7.32	R^1R^1 or R^1r'	5.35	12.7
R^1R^1 or R^1r'	6.69	R^1R^1 or R^1r'	5.60	12.3
			Mean	12.5

* Antibody nitrogen in 10^{-2} μg N. The data have not been corrected for the nitrogen uptake of $Rh_0(D)$ negative red cells. Total nitrogen content of the I^{131} anti-$Rh_0(D)$ eluate was 3.96 μg N and 1 ml of a 1 per cent cell suspension was used for each reaction.

minutes was 10.33 ± 5.36 per cent (Table IX). This increase in antibody uptake following the twofold increase in reaction time is consistent with the kinetics of this reaction (7).

Frequency distribution of antibody nitrogen bound to $Rh_0(D)$ positive red cells. At the top of Figure 2 is a plot of the antibody nitrogen bound to 0.01 ml centrifuged red cells for a population of 47 $Rh_0(D)$ positive, rh′(C) negative red cells. On the basis of inspection the distribution can be divided into two groups by using the 3.2×10^{-2} μg N value. One group then consists of 37 different red cell samples with a mean value of $2.44 \pm 0.27 \times 10^{-2}$ μg N per 0.01 ml RBC, and the other group consists of 10 different red cell samples with a mean of 3.95 ± 0.34. The group of cells with the higher nitrogen values has an average nitrogen that is 1.62 times greater than that found in the group with the lower nitrogen values.

The two lower panels in Figure 2 present the distribution of two of the three common $Rh_0(D)$ positive, rh′(C) negative phenotypes. There are 14 red cell samples with an R^2R^2 or R^2r'' phenotype, 29 with an R^2r or R^2R^0 phenotype and 4 with an R^2r or R^0R^0 phenotype. There are insufficient numbers in each category to derive independent statistical values, but it is apparent that the distribution of antibody nitrogen values found in these different phenotypes does not differ from the distribution obtained by pooling all of the rh′(C) negative, $Rh_0(D)$ positive phenotypes.

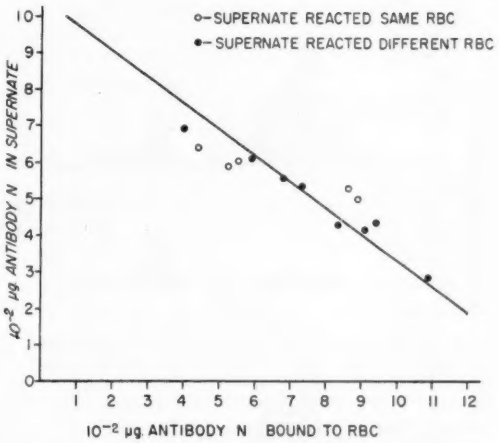

FIG. 1. RELATIONSHIP BETWEEN ANTIBODY N IN SUPERNATE AND ANTIBODY N BOUND TO RED CELLS. The antibody nitrogen bound to the $Rh_0(D)$ positive red cell is shown on the abscissa and the antibody nitrogen that was recovered in the supernate is plotted on the ordinate. The total quantity of antibody recovered in these supernate studies was unaffected by the Rh phenotype of the red cell that was used to test the supernate.

The frequency distribution of antibody nitrogen values in a population of 152 rh′(C) and $Rh_0(D)$ positive red cells is shown at the top of Figure 3. The bimodal distribution is evident but it is not as clearly defined as that found with the rh′(C) negative red cell. If 2.3×10^{-2} μg N is used to divide the two groups, one group has a mean value

TABLE IX

*Effect of reincubation on the quantity of antibody bound to red cells**

Probable red cell genotype	Nitrogen bound 10^{-2} μg N per 0.01 ml centrifuged RBC: After incubation for 60 min	After second incubation for 60 min	Per cent increase in nitrogen bound to red cells
R^1r or R^1R^0	1.76	1.80	2.3
R^1r or R^1R^0	2.10	2.28	8.6
R^1r or R^1R^0	3.54	3.79	7.1
R^1r or R^1R^0	2.17	2.34	7.8
R^1R^1 or R^1r'	3.31	3.59	8.5
R^1r or R^1R^0	1.53	1.88	22.9
R^1r or R^1R^0	2.55	2.87	12.6
R^1R^2 or R^1r''	3.65	4.13	13.2
R^2r or R^2R^0	2.87	3.18	10.1
R^1R^1 or R^1r'	2.68	2.96	10.5
		Mean	10.33 ± 5.36

* Red cells were washed and assayed for I^{131} after the first 60 minute incubation period and then were re-reacted with additional I^{131} anti-$Rh_0(D)$ for a second 60 minute period. One ml of the I^{131} anti-$Rh_0(D)$ with a total nitrogen content of 3.96 μg was used in both reactions and 0.5 ml of a 5 volumes per cent cell suspension was employed. The data have not been corrected for the nitrogen uptake of $Rh_0(D)$ negative red cells.

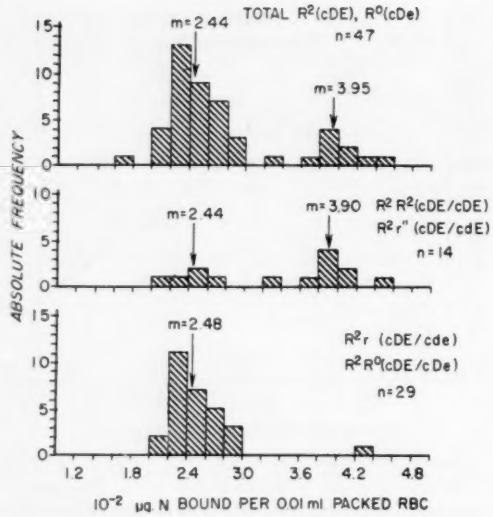

FIG. 2. DISTRIBUTION OF ANTIBODY N BOUND TO $Rh_0(D)$ POSITIVE, rh'(C) NEGATIVE RED CELLS. The bar graph at the top of this figure presents the findings for the total group of rh'(C) negative red cells. The two lower bar graphs are the results plotted separately for two of the common phenotypes.

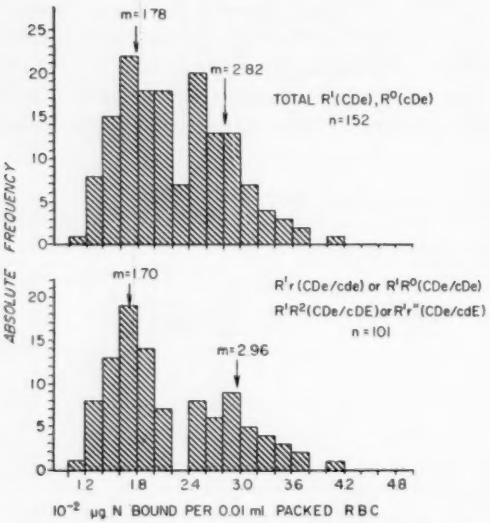

FIG. 3. DISTRIBUTION OF ANTIBODY N BOUND TO $Rh_0(D)$ AND rh'(C) POSITIVE RED CELLS. The bar graph at the top of the figure presents the findings for the total group of $Rh_0(D)$ and rh'(C) positive red cells. The lower plot presents the data obtained on only the heterozygous rh'(C) red cell samples.

of $1.78 \pm 0.03 \times 10^{-2}$ µg N per 0.01 ml RBC and the other group 2.32 ± 0.05. These values are significantly less than the corresponding values for the rh'(C) negative red cells, 2.44 and 3.95. The effect of the homozygous rh'(C) positive red cells on this distribution is shown at the bottom of Figure 3. The homozygous rh'(C) positive red cells have been excluded from this graph so that only the values for the heterozygous rh'(C) positive red cells are shown. A total of 101 different

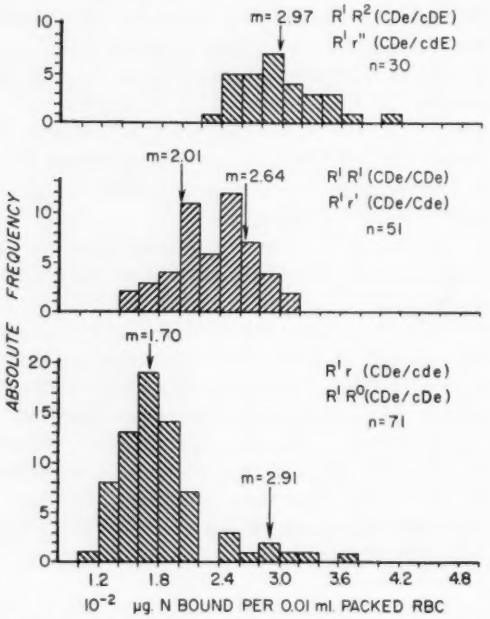

FIG. 4. DISTRIBUTION OF ANTIBODY N BOUND TO $Rh_0(D)$ AND rh'(C) POSITIVE RED CELLS. The results obtained for each of the three common $Rh_0(D)$ and rh'(C) positive phenotypes are shown in each bar graph. The data used to plot the bar graph at the top of Figure 3 were obtained by combining these three groups.

red cell samples has been plotted of which 71 had an R^1r or R^1R^0 phenotype and 30 had an R^1R^2 or R^1r' phenotype. In this case 62 of the 101 cell samples have a mean value of 1.70 ± 0.03 and 39 have a mean value of 2.96 ± 0.07, 10^{-2} µg N per 0.01 ml RBC.

The distortion in the bimodal distribution introduced by the rh'(C) red cell is shown by the data plotted in Figure 4. In this plot the three common rh'(C) positive red cell phenotypes are shown individually. At the top of the figure are 30 R^1R^2

TABLE X

Chromosome frequencies

Chromosome	Calculated from total sample* (n = 275)	Calculated from cells studied† (n = 210)	Reported by Race and Sanger (1)
r (cde)	0.3133	0.2070	0.3886
r′ (Cde)	0.0114	0.0012	0.0098
r″ (cdE)	0.0170	0.0012	0.0119
R^0 (cDe)	0.0224	0.0418	0.0257
R^1 (CDe)	0.4596	0.4873	0.4076
R^z (CDE)	0.0038	0.0005	0.0024
R^2 (cDE)	0.1725	0.2611	0.1411

* Includes the red cell samples from the family studies and some samples that had been phenotyped but which had not been reacted with the I^{131} anti-Rh_0(D).

† Excludes the family study samples and includes only the red cell samples that were reacted with the I^{131} anti-Rh_0(D).

or R^1r'' phenotypes; the middle plot shows 51 R^1R^1, or R^1r' phenotypes; and the graph at the bottom presents the findings on 71 R^1r or R^1R^0 red cell samples. The homozygous rh′(C) positive red cells (middle graph, Figure 4) show the poorest separation into two groups. If this distribution is divided arbitrarily into two groups by the 2.3×10^{-2} μg N value the standard error of the mean for each group can be determined and the two groups can be compared by the *t* test. Under these conditions the two groups are significantly different, $p < 0.05$. The heterozygous rh′(C) positive red cells show a better separation into two groups, one group with a mean nitrogen value of 1.70 and the other group with a mean nitrogen value of 2.91.

Chromosome frequencies. The chromosome frequencies for these data were calculated using the method described by Race and Sanger (1). Unfortunately, valid chromosome frequencies cannot be derived from these results since the calculations are not based on a random sample of Caucasian red cells. The red cells used for these studies were derived from a special population (blood donors), only Rh_0(D) positive red cells were selected and during the course of the study a conscious effort was made to exclude the rh′(C) positive red cells. The effect of these selection criteria on the chromosome frequencies is shown in Table X. If all the red cell samples, including the family studies, are used for the calculations the chromosome frequencies are close to those reported by Race and Sanger (1). This agreement, however, is spurious because the exclusion of the Rh_0(D) negative red cells from the data was compensated for by the family studies which contained an excess of Rh_0(D) negative cells. The bias introduced by the devices used to select the red cells for this study is evident when the chromosome frequencies are derived from only the red cells that were reacted with the I^{131} anti-Rh_0(D) and from which the family studies had been excluded. These values found

TABLE XI

Family studies

Father		Mother		Children	
Phenotype	N bound*	Phenotype	N bound*	Genotype	N bound*
R^1r'' (CDe/cdE)		rr (cde/cde)	0.17	R^1r (CDe/cde)	2.22
R^1R^2 (CDe/cDE)	3.41			R^1r (CDe/cde)	2.48
R^2R^2 (cDE/cDE)		r″r (cdE/cde)	0.15	R^2r'' (cDE/cdE)	1.86
R^2r'' (cDE/cdE)	2.96			R^2r'' (cDE/cdE)	1.50
R^1r (CDe/cde)	1.47	r″r (cdE/cde)	0.13	rr(cde/cde)	0.26
R^1R^0 (CDe/cDe)					
R^1r' (CDe/Cde)	†	r′r (Cde/cde)	0.10	R^1r' (CDe/Cde)	1.49
R^1r (CDe/cde)				r′r (Cde/cde)	0.14
R^1r (CDe/cde)	1.81	R^1r (CDe/cde)	1.90	rr (cde/cde)	0.16
R^1R^0 (CDe/cDe)		R^1R^0 (CDe/cDe)			

* 10^{-2} μg N per 0.01 ml centrifuged red cells.

† Father not available for study.

TABLE XII

Nonreacting $Rh_0(D)$ positive red cells

Phenotype	10^{-2} μg N bound to 0.01 ml red cells*
O, r''r (cdE/cde), D^u pos.	0.17
O, r'r (Cde/cde), D^u pos.	0.24
B, R^0r (cDe/cde) or R^0R^0 (cDe/cDe)	0.53
O, R^0r (cDe/cde) or R^0R^0 (cDe/cDe)	0.32

* The mean nitrogen bound to $Rh_0(D)$ negative red cells has not been subtracted from the values in the table.

in the second column of Table X differ considerably from the values expected for a Caucasian population (last column, Table X).

Family studies. The data obtained by studying the red cells of five families with eight children are shown in Table XI. The first four families involve $Rh_0(D)$ incompatible mating in which the father is $Rh_0(D)$ positive and the mother is $Rh_0(D)$ negative. Of the seven children that were studied, two were $Rh_0(D)$ negative and five were $Rh_0(D)$ positive. Four of the five heterozygous $Rh_0(D)$ positive red cell samples obtained from these children took up a quantity of antibody nitrogen that was within the lower peak of the bimodal distribution found in the population study. The R^1r red cells of one child had a value, 2.48×10^{-2} μg N, that was slightly greater than the 2.3×10^{-2} value which was used to divide the distribution of this particular phenotype into two groups.

In one family both the mother and child were $Rh_0(D)$ negative and the father was $Rh_0(D)$ positive. The antibody nitrogen bound to the father's heterozygous $Rh_0(D)$ red cells was within the lower nitrogen value group. In the last family shown on Table IV the child was $Rh_0(D)$ negative and the parents were $Rh_0(D)$ positive. The heterozygous $Rh_0(D)$ positive red cells of both parents had nitrogen values that were within the lower nitrogen peak of the bimodal distribution.

Reactivity of the red cell used to immunize. Two different red cells involved in the immunization of the antiserum donor were available for testing with the I^{131} anti-$Rh_0(D)$. One of the cells was the husband's red cell whose phenotype was A, R^1R^1 (CDe/CDe) or R^1r' (CDe/Cde). The other cell, A_1, R^0r (cDe/cde) or R^0R^0 (cDe/cDe), was the red cell used to reimmunize the donor. Both of these cells were reacted with the I^{131} anti-$Rh_0(D)$ to see if their reactivity was consistent with that found with other $Rh_0(D)$ positive red cells of the same Rh phenotype. The husband's cells took up 2.70×10^{-2} μg N per 0.01 ml RBC and the red cells used as the "booster" antigen took up 2.26. Both of these values fell within the range of values obtained with other red cells of similar Rh phenotype.

$Rh_0(D)$ variants. Of the 278 different $Rh_0(D)$ positive red cells studied, four different cell samples were found that did not take up significant quantities of the I^{131} anti-$Rh_0(D)$. Two of these cell samples were D^u positive and two were D^u negative. The phenotypes and quantity of anti-

TABLE XIII

*Assignment of antibody nitrogen values**

Phenotype	Heterozygous $Rh_0(D)$		Homozygous $Rh_0(D)$	
	Nitrogen†	No.‡	Nitrogen†	No.‡
R^2R^2 or R^2r''	2.44 ± 0.08	5	3.90 ± 0.11	9
R^2r, R^2R^0, or R^0r''	2.48 ± 0.05	28	4.38	1
R^0r or R^0R^0	2.18	4		
Total R^2, R^0	2.44 ± 0.04	37	3.95 ± 0.11	10
R^1r, R^1R^0, or R^0r'	1.70 ± 0.03	62	2.91 ± 0.16	9
R^1R^1 or R^1r'	2.02 ± 0.04	23	2.64 ± 0.04	28
R^1R^2, or R^1r'' or R^2r'		0	2.97 ± 0.08	30
Total R^1, R^2	1.78 ± 0.03	85	2.82 ± 0.05	67

* The table is based on red cells derived from 199 Caucasian individuals.

† The nitrogen value represents the mean and standard error of the mean in units of 10^{-2} μg N bound to 0.01 ml RBC.

‡ Represents the number of different red cell samples of the phenotype indicated which were used to determine the mean nitrogen value.

body nitrogen taken up by these four red cell samples are shown in Table XII. These four samples took up only 10 to 15 per cent of the nitrogen that was bound to the other $Rh_0(D)$ positive red cell samples, and in all cases, except one, these cells took up more I^{131} than did the $Rh_0(D)$ negative red cells. All four cell samples were negative by the indirect antiglobulin test after reaction with the I^{131} anti-$Rh_0(D)$. The original serum and the I^{131}-γ-globulin fraction before the absorption-elution procedure, however, gave positive indirect antiglobulin reactions with these cells. Although an antibody to the $Rh_0(D)$ antigen present in these red cells was available in the original antiserum, it apparently was lost during the stroma absorption and elution procedure.

Assignment of genotype to antibody values. Table XIII presents a summary of the $Rh_0(D)$ red cell antigen content of the common $Rh_0(D)$ genotypes. These values were derived from the study of 199 samples of Caucasian red blood cells and the genotype was assigned to these values using the data obtained from the family studies.

DISCUSSION

The $Rh_0(D)$ antigen content of the red cell has been estimated from the quantity of I^{131} incomplete anti-$Rh_0(D)$ bound to the red cell. A bimodal distribution of the red cell $Rh_0(D)$ content was found in a population of 199 different $Rh_0(D)$ positive red cell samples. Family studies indicate that the two groups of red cells are related to the $Rh_0(D)$ genotype of the individual from whom the cells were obtained. The segregation into two distinct populations was most evident for the red cells free of the rh′(C) antigen. The rh′(C) positive $Rh_0(D)$ containing red cells differed from the rh′(C) negative cells in two ways. There was an absolute decrease in total $Rh_0(D)$ antigen content as defined by the quantity of I^{131} anti-$Rh_0(D)$ bound to these cells and the cells were not as sharply separated into two groups. The homozygous rh′(C) red cells showed the poorest separation into two groups (middle bar graph in Figure 4). The $Rh_0(D)$ homozygous red cell in this case had a greater decrease in $Rh_0(D)$ content than did the heterozygous $Rh_0(D)$ cell. The nonproportional decrease of the $Rh_0(D)$ antigen in the two genotypes resulted in overlapping of the two distributions, so that a separation of the two $Rh_0(D)$ genotypes was not as clearly defined as that observed in the other Rh genotypes.

The suppressive or inhibiting effect of rh′(C) on the quantitative expression of the $Rh_0(D)$ property has been recognized by many investigators (*20–22*). This study is inadequate to explain the effect of the rh′(C) antigen on the quantity of I^{131} anti-$Rh_0(D)$ bound to rh′(C) positive red cells. The decrease in antibody uptake by these cells may be due to an immunological effect exerted by the rh′(C) antigen or the effect may be due to a genetic mechanism. Current genetic theories postulate that the rh′(C) effect represents a decreased elaboration of the $Rh_0(D)$ antigen resulting from either competition for a common substrate (*22*) or from some interaction between alleles at the Rh locus (*21*). The effect of rh″(E) on the quantity of I^{131} anti-$Rh_0(D)$ bound to rh″(E) positive red cells cannot be evaluated adequately since only four samples of R^0r (cDe/cde) or R^0R^0 (cDe/cDe) red cells were studied. The precise analysis of the interaction of the $Rh_0(D)$, rh′(C) and rh″(E) antigens must await more detailed family studies and a technique for the direct determination of the $Rh_0(D)$ red cell antigen content.

An effort was made to critically evaluate and experimentally control the two obvious limitations associated with the use of I^{131} anti-$Rh_0(D)$ to estimate the red cell $Rh_0(D)$ antigen content. One is the experimental error resulting from the techniques used to handle particulate suspensions (cells) quantitatively and the other is the immunochemical variability or heterogeneity of the $Rh_0(D)$ antigen.

The errors introduced into the results consist of those due to the experimental procedures and those that may result from the use of the hematocrit as an index of the number of cells present in the cell suspension. The assumption that the hematocrit is directly proportional to the number of cells implies that there is no change in cell volume during the study and that there is uniformity of red cell volume from one individual to the next. The results obtained in the storage experiments (Table V) indicate that there was no demonstrable change in cell volume during storage and that the reactivity of the antigen sites was unaffected by

either the conditions of storage or the procedures used in these studies.

The errors due to the experimental procedures can be evaluated from the variances listed in Table IV. These results indicate that less than one-fifth of the total variance is due to the procedure in which the red cells are reacted with the I^{131} anti-$Rh_0(D)$. The major source of error is associated with the determination of the hematocrit of the red cell suspension that is used in the reaction with the labeled antibody.

A reliable estimate of the number of antigen sites which is based on the quantity of cell-bound antibody requires that the reaction be in antibody excess. Insufficient antibody to saturate all the available antigen sites in the red cell suspension would lead to an underestimation of the antigen sites. The presence of antibody excess in these experiments was determined in two ways; in one case, the supernates from $Rh_0(D)$ positive cells were tested for free antibody, and in the other method, the sensitized, presumably saturated red cells, were re-reacted with additional antibody. The results obtained by both techniques showed that excess antibody was present (Tables VIII and IX).

The quantity of I^{131} taken up by $Rh_0(D)$ negative red cells is one of the possible indices of the specificity of the I^{131} bound to $Rh_0(D)$ positive red cells. The amount of I^{131} taken up by the $Rh_0(D)$ negative red cells was less than 6 per cent of the I^{131} bound to $Rh_0(D)$ positive red cells and was independent of the Rh or ABO phenotype (Table VII). Although previous absorption of the I^{131} anti-$Rh_0(D)$ negative red cells does not affect the content of anti-$Rh_0(D)$, the nature of the I^{131} protein bound to the $Rh_0(D)$ negative red cells has not been defined by these experiments.

The specificity of the anti-$Rh_0(D)$ was also evident in the experiments carried out on the $Rh_0(D)$ positive red cell supernates. A constant quantity of antibody was recovered when the antibody found in the supernate was added to the antibody taken up by the original red cell. This was true irrespective of the type of $Rh_0(D)$ positive red cell used to absorb the antibody remaining in the supernate (Table VIII and Figure 1). The total recovery of antibody was, in addition, independent of the amount removed by the first $Rh_0(D)$ positive red cell. These findings indicate that the $Rh_0(D)$ quality defined by the I^{131} anti-$Rh_0(D)$ was specific and consistent with a single anti-$Rh_0(D)$ which was absorbed stoichiometrically by a large number of $Rh_0(D)$ positive red cells.

The heterogeneity of the $Rh_0(D)$ antigen has been documented by many investigators (1). Four $Rh_0(D)$ positive red cell samples that did not react with the I^{131} anti-$Rh_0(D)$ were found in this series. The occurrence of these cells indicates that the antibody preparation used in this study was selected for a given quality or qualities of the $Rh_0(D)$ antigen by the techniques employed to obtain the antibody. Two independent operations served to define the $Rh_0(D)$ specificity of the I^{131} anti-$Rh_0(D)$. Specificity was determined by the nature of the antigens that were used to immunize the donor (multiple pregnancies and intentional re-immunization) and by the $Rh_0(D)$ antigen content of the red cell stroma used to concentrate the I^{131} anti-$Rh_0(D)$. The use of a pool of red cell stroma derived from many $Rh_0(D)$ positive red cell samples was an attempt to minimize the selective role of the stroma absorption and elution technique. In spite of these precautions it is evident that the I^{131} anti-$Rh_0(D)$ used in this study did not contain antibodies to all the $Rh_0(D)$ antigens.

Additional evidence for the heterogeneity of the $Rh_0(D)$ antigen was the distribution of the $Rh_0(D)$ antigen content in Negro red cells. The $Rh_0(D)$ antigen content in these red cells showed a distribution different from that found in Caucasian red cells. Inadequate numbers of such cells have been studied to determine if the difference noted is significant. Such a difference is consistent with the recognized difference in the frequency of many of the blood group antigens in the Negro (*23*).

The gene-product determined by these studies (blood group antigen) is restricted to the configuration of the antibody combining site on a mucopolysaccharide molecule if the $Rh_0(D)$ antigen resembles the ABO antigens (*24*, *25*). Although a high degree of specificity exists for this site, it probably represents only a very small fraction of the total molecule (*26*, *27*). As a result, only a fragment of the gene-controlled molecule is visualized so that no inference concerning the rest of the molecule can be drawn from these studies. It is conceivable that the antigenic configurations responsible for the different blood

group specificities are all found on a common molecule. The concept of such a basic blood group nucleus has been postulated for the $Rh_0(D)$ antigens (28). If experimental support can be obtained for this concept then the basic blood group nucleus would represent a molecule whose synthesis is dependent on many genes. The blood group antigens would then resemble other gene-controlled molecules, such as hemoglobin, which are believed to be under the control of several different genes (29). This resemblance, however, may be superficial in that the hemoglobin loci control primary protein structure (30), whereas the immunochemical specificity of the blood group substances could be due to changes confined to only the secondary protein structure.

An estimate of the number of antigen molecules or sites can be derived from these data if certain assumptions are granted. If the specific antibody iodinates to the same extent as the total γ-globulin fraction (16) and if 1.6×10^5 is used for the molecular weight of the $Rh_0(D)$ iso-antibody, the homozygous $Rh_0(D)$ cell contains about 10,300 antigen sites and the heterozygous cell about 6,400 antigen sites. For the rh′ (C) positive cell these values are 7,400 and 4,600, respectively. These values, although larger, are of the same order of magnitude as those that have been estimated with the techniques employed in previous studies (4–7). It will be of interest to determine the quantity of antibody bound to an individual cell to check the validity of these values which are based on averages.

The results obtained with the application of these techniques to the $Rh_0(D)$ antigen indicates that the use of an I^{131} labeled iso-antibody offers an exquisitely sensitive method for studying the distribution, quantity and specificity of the blood group antigens. The ability to quantitatively evaluate these parameters provides a means of investigating the genetic and immunochemical interrelationships of the substances of the blood groups. An obvious clinical application of this study has been made to the problem of hemolytic disease of the newborn due to Rh incompatibility. The genotype of $Rh_0(D)$ positive fathers has been determined using the data of this study so that a prognosis could be made with regard to future pregnancies.

CONCLUSIONS

1. An estimate of the $Rh_0(D)$ antigen content of 199 different Caucasian red cells was obtained from the quantity of I^{131} trace labeled incomplete anti-$Rh_0(D)$ bound to the red cells.

2. The validity of the technique was evaluated by defining the experimental errors, the $Rh_0(D)$ specificity of the I^{131} anti-$Rh_0(D)$, and the quantitative relationship between volume of red cells, quantity of I^{131} anti-$Rh_0(D)$ bound to the red cells, and the I^{131} anti-$Rh_0(D)$ that could be demonstrated in the supernate.

3. The quantity of I^{131} anti-$Rh_0(D)$ bound to rh′(C) negative, $Rh_0(D)$ positive red cells showed a bimodal distribution. The group of cells with the higher antigen content (1.6 times) was identified with the homozygous state [$Rh_0(D)$, $Rh_0(D)$] and the other group with the heterozygous state [$Rh_0(D)$], rh(d), by using genetically defined heterozygous $Rh_0(D)$ red cells obtained from family studies.

4. The rh′(C) positive red cells took up only 70 to 73 per cent of the I^{131} anti-$Rh_0(D)$ that was bound to the rh′(C) negative cells. The decrease in quantity of bound I^{131} anti-$Rh_0(D)$ was uniform for both $Rh_0(D)$ genotypes, except for the cells homozygous for the rh′(C) antigen. This effect of the rh′(C) antigen on the $Rh_0(D)$ content may be apparent (immunochemical basis) or absolute (genetic mechanism).

5. Evidence for the heterogeneity of the $Rh_0(D)$ antigen was obtained. As a result the data presented apply only to the $Rh_0(D)$ antigen defined by the particular I^{131} anti-$Rh_0(D)$ used in this study.

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CHEMICAL CHARACTERIZATION OF CARDIAC MYOSIN FROM NORMAL DOGS AND FROM DOGS WITH CHRONIC CONGESTIVE HEART FAILURE

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The size and chemical nature of the myosin molecule have been studied extensively in enzyme preparations obtained from skeletal muscle (1–7), but a paucity of data is available on cardiac myosin. Furthermore, different values for the molecular weight of cardiac myosin have been reported. Olson (8) reported a value of 223,000 for the molecular weight of cardiac myosin whereas Gergely and Kohler (9) presented evidence that myosin from cardiac muscle has a molecular weight of 500,000, which is similar to that obtained during the most recent observations on skeletal muscle myosin (4–7).

It has been suggested that the cardiac myosin molecule is altered in cardiac failure to form a stable aggregate of the basic monomer and that abnormal myosin prevents the formation of normal cardiac actomyosin (8). On the contrary, considerable evidence has been adduced that actomyosin from the failing heart is normal (10). The present observations were undertaken to determine the molecular size and chemical nature of normal cardiac myosin and to compare these data with results obtained on cardiac myosin from dogs with chronic congestive heart failure.

METHODS

Muscle was obtained from the hearts of 12 normal dogs, 11 dogs with right-sided cardiac failure secondary to surgically-induced tricuspid insufficiency and pulmonic stenosis, one dog with naturally occurring right- and left-sided congestive failure and 3 dogs with chronic ascites produced by constriction of the thoracic inferior vena cava. The techniques used for the surgical procedures have been described previously (11–13). On the day of sacrifice, studies were conducted to determine the physiological state of the animals. All measurements of cardiovascular pressures were made in trained unanesthetized dogs; the methods have been described previously (13). The methods for Na balance measurements are described elsewhere (11, 12); Na intake was 60 mEq per day.

Cardiac muscle was obtained by anesthetizing the animals lightly with intravenous sodium pentobarbital, opening the chest quickly and excising the beating heart. The heart was washed with cold water and placed in a container with chipped ice. The right and left ventricular walls were separated from the septum. Since only right-sided heart failure was present in dogs with tricuspid insufficiency and pulmonic stenosis (13), muscle from the right and from the left ventricles of these dogs was extracted separately for myosin. The left ventricle only was used for preparation of myosin from the one dog with naturally occurring right- and left-sided cardiac failure and from the dogs with constriction of the thoracic inferior vena cava. Myosin was extracted from combined right and left ventricles of normal dogs.

Myosin was prepared by the method of Gergely (14). The extraction of myosin was begun immediately after obtaining heart muscle. Since the yield of myosin was low, all of the right or left ventricular muscle masses was used. To obtain enough myosin for diffusion measurements, in some instances the right ventricles from 2 dogs were combined for extraction and the left ventricles from the same animals were extracted as one preparation. The procedure to be described for the extraction of myosin applies for a quantity of 75 g of cardiac muscle. If less muscle was available, the volumes of the extracting media were adjusted accordingly. The entire extraction procedure was carried out at 0° to 5° C.

Seventy-five g of heart muscle was ground with a meat grinder and extracted with 300 ml of a salt solution by stirring for 45 minutes. The salt solution contained 0.3 M KCl, 0.2 M potassium phosphate buffer (pH 6) and 0.02 M sodium pyrophosphate; the final pH of the solution was adjusted to 6.2 with KOH. The muscle extract was squeezed through cheesecloth and the residue discarded. The extract was centrifuged at 2,000 rpm in a Lourdes model AT centrifuge for 30 minutes. To precipitate the myosin, 10 volumes of glass distilled water were added to each volume of the supernatant. The precipitate of myosin was allowed to settle for at least 1 hour and the supernatant was siphoned off. The remaining myosin solution was centrifuged at 8,000 rpm in the Lourdes centrifuge for 10 minutes. The supernatant was discarded and the residue was dissolved in 2.4 M

TABLE I

Physiological data on experimental animals at the time of sacrifice

Dog no.	Initial body wt	Time after last operation	Duration of ascites	Volume of ascites	Renal Na excretion	Plasma Na	Plasma K	Mean right atrial pressure C†	Mean right atrial pressure E†	LVEDP* C	LVEDP* E
	kg	*days*	*days*	*L*	*mEq/day*	*mEq/L*	*mEq/L*	*mm water*		*mm water*	
Dogs with right-sided failure produced by tricuspid insufficiency and pulmonic stenosis											
1	18.3	91	80	8.00	4.3	151	4.2	50	240	95	28
2	21.5	118	88	0.20	15.4				175		42
3	20.5	195	192	4.90	39.0	155	3.9	76	190	81	67
4	17.0	144	132	1.65		142	4.3	65	330		
5	19.0	55	51	2.80		144	4.3	98	140	119	99
6	18.9	86	79	0.61	5.9	141	4.7	60	144	75	55
7	18.5	86	68	3.00	2.5	137	3.9	40	152	108	65
8	25.0	69	68	3.50		149	4.3		275		108
9	20.5	88	85	3.70	1.4	152	4.7		205		
10	19.0	74	68	0.01	32.6	147	3.5		165		
11	18.6	75	71	0.10	50.5	148	3.8		180		
$\bar{X}$	19.8	98.2	89.2	2.59	19.0	147	4.2	65	199	96	66
Dog with naturally occurring right- and left-sided heart failure											
1	41.0		150	1.20		153	3.6		230		210
Dogs with constriction of the thoracic inferior vena cava											
1	17.9	92	88	3.00	1.9	143	4.1		280‡		75
2	18.0	74	61	4.50	7.5	144	3.8		175‡		75
3	22.0	166	165	2.50	0.6	140	4.7		165‡		63

* LVEDP is the abbreviation for left ventricular end-diastolic pressure.
† C and E are abbreviations for control and experimental, respectively.
‡ Inferior vena caval pressures instead of right atrial pressures.

KCl. The solution of myosin was diluted to 0.6 M KCl and enough 0.6 M KCl was added to make 100 cc. Potassium carbonate was added to adjust the pH to 7.5 and the solution was stirred overnight with a magnetic stirrer. The preparation was centrifuged at 16,500 rpm for 30 minutes, in the Lourdes centrifuge. The myosin was precipitated for the second time by adding again 10 volumes of glass distilled water to each volume of myosin solution. The myosin was allowed to settle and the supernatant was siphoned off and discarded. The myosin solution was centrifuged at 8,000 rpm for 10 minutes. The residue was dissolved in 2.4 M KCl, diluted to 0.6 M KCl and sufficient 0.6 M KCl was added to make 100 cc. The pH was adjusted to 7 with potassium carbonate. The solution was diluted to 0.3 M and centrifuged at 8,000 rpm for 10 minutes in the Lourdes centrifuge. The third precipitation was carried out by further dilution to a 0.06 M solution. The myosin solution was allowed to settle overnight. The supernatant was siphoned off and the remainder was centrifuged at 8,000 rpm for 10 minutes. The residue was dissolved in 2.4 M KCl and the concentration was adjusted to 0.6 M KCl. The myosin preparation was centrifuged at 16,500 rpm for 30 minutes in the Lourdes centrifuge. The supernatant myosin solution was decanted from the pellet and centrifuged in a Spinco model L ultracentrifuge for 4 hours at 30,000 rpm. The final myosin solution was decanted from the pellet. The yields ranged from 75 to 200 mg per 100 g of cardiac muscle.[1]

[1] No attempt was made to compare the yields of myosin from normal and failing cardiac muscle. The aim of the extraction procedure was to achieve purity of preparation and the large losses of myosin which occurred would make any attempt at quantitation meaningless.

Measurements of sedimentation velocity of cardiac myosin were made with a Spinco model E analytical ultracentrifuge. Two samples were centrifuged simultaneously by use of a wedge cell and a plain cell. Either two different concentrations of myosin or one concentration of myosin with added ATP and $MgCl_2$ and the same preparation and concentration of myosin without these compounds were centrifuged. The final concentrations of ATP and $MgCl_2$ were 5×10^{-3} M and 10^{-2} M, respectively. Kel F centerpieces were used to prevent denaturation of the protein. Ultracentrifugation [2] was performed at 59,780 rpm or 250,000 G at a temperature of 7° C; the bar angle was 60°. Actual sedimentation constants were corrected to 20° C and to water as a solvent by the standard formula (15). A value of 0.728 was used for the partial specific volume of myosin.

Diffusion measurements were made in an Aminco model B electrophoresis apparatus at 6° C, using carefully selected cells and the Raleigh interferometer in the manner described by Longsworth (16). The myosin solutions were dialyzed for 18 to 20 hours against 0.6 M KCl buffered to pH 7.6 with phosphate. Diffusion coefficients were corrected to 20° C and to water as a solvent. Sedimentation constants were determined on all but one of the myosin preparations used for diffusion measurements.

Viscosity was measured with a size II Ubbelohde viscosimeter with an outflow time for 0.6 M KCl of 100 seconds. All viscosity measurements on myosin were made within the first day or two after completion of the

[2] Slight variation in the $S_{20,w}$ has been reported with different rotor speeds (3). Ultracentrifugation was performed at 59,780 rpm because measurements are more accurate with this rotor speed than with slower rates of sedimentation for large molecules such as myosin (3, 15).

preparation. Myosin was dissolved in 0.6 M KCl and measurements were made in a constant temperature water bath at 22.5° ± 0.02° C. Multiple determinations on a myosin preparation were made by repeated dilution of the myosin solution and subsequent measurement of the viscosity. The relative viscosity (η_{rel}) was determined as the ratio of the outflow time for the solution of myosin to that of 0.6 M KCl; η_{sp} is equal to η_{rel}^{-1}.

The protein concentration of the myosin solutions was determined by micro-Kjeldahl analysis.

RESULTS

Physiological studies of experimental animals. The results are presented in Table I. The 11 dogs with tricuspid insufficiency and pulmonic stenosis showed evidence of pure right-sided congestive failure. Mean right atrial pressure was markedly elevated in every instance, whereas left ventricular end-diastolic pressure was normal. Ascites was present in all dogs but Na excretion varied from 1.4 to 50.5 mEq per day. The ratio of right ventricular to left ventricular weight was 0.93 in comparison with the normal value of 0.51 (13) ($p < 0.001$). Other previously reported data (13) on heart and lung weights in dogs with cardiac failure secondary to tricuspid insufficiency and pulmonic stenosis showed no evidence of left ventricular failure or hypertrophy. The average duration of life after the second operation (production of pulmonic stenosis) was 98 days, whereas the average duration of ascites was 89 days.

The one animal with naturally occurring congestive heart failure showed evidence of both right and left ventricular hypertrophy and failure. The filling pressures in both the right and the left heart were elevated. The heart was massively enlarged and weighed 502 g. The lungs weighed almost 2.5 times the average normal value of 9.15 ± 1.30 g per kg (13) and showed microscopic evidence of chronic passive congestion. The liver showed evidence histologically of chronic congestion and presented the characteristic nutmeg appearance grossly.

In addition to the normal animals, three dogs with thoracic caval constriction and chronic ascites provided control material. These animals were not in cardiac failure but they showed essentially the same alterations in salt and water metabolism and the protein depletion observed in experimental heart failure (11–13). Consequently, cardiac muscle from the dogs with caval constriction provided a control for evaluation of possible changes in failing heart muscle which might be secondary to altered electrolyte or protein metabolism. Data on their physiological state are presented in Table I. An additional finding of interest in these animals is the presence of both right and left ventricular atrophy which has been reported elsewhere (13).

Sedimentation velocity studies of myosin. The sedimentation of myosin was observed in enzyme preparations made from the combined right and left ventricles of normal dogs. Myosin from separate right and left ventricles of dogs with right heart failure secondary to tricuspid insufficiency and pulmonic stenosis was studied while left ventricular myosin only was obtained from dogs with constriction of the thoracic inferior vena cava. In addition, studies were made on myosin from the left ventricle of a dog with naturally occurring right- and left-sided failure.

Qualitatively the sedimentation patterns were identical for myosin from all sources of cardiac muscle. The typical sedimentation pattern consisted of a single peak which suggested homogeneity of the myosin preparation (Figure 1). In a few instances, a very small boundary which sedimented faster than the principal myosin component was observed. The magnitude of this boundary was not sufficient to produce sedimentation constants which were different from those obtained in myosin preparations with only one sedimentation boundary. Addition of ATP immediately before centrifugation did not influence the sedimentation pattern or rate of movement of myosin (Figure 1).

Quantitative data on sedimentation velocity are presented in Figure 2. The $S_{20,w}$ at zero concentration for myosin from normal heart muscle was 6.17×10^{-13} seconds. The slopes of the least squares straight lines and the y intercepts (Figure 2) for the myosin from failing right or left ventricles, from the nonfailing left ventricle of dogs with tricuspid insufficiency and pulmonic stenosis, and from the atrophic left ventricle of dogs with thoracic caval constriction were not statistically different from the values for normal cardiac myosin. Both the slope of the line and the y intercept for myosin from the left ventricle of dogs with tricuspid insufficiency and pulmonic stenosis were

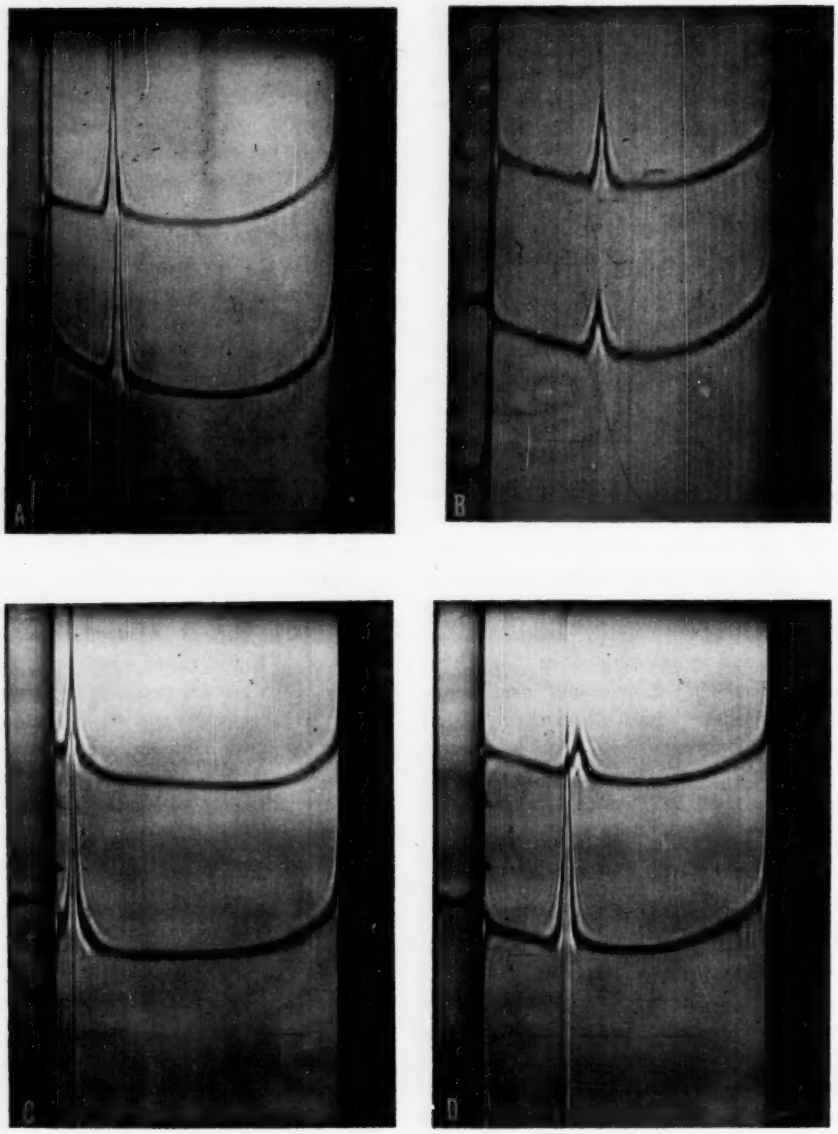

FIG. 1. ULTRACENTRIFUGAL SEDIMENTATION PATTERNS OF CARDIAC MYOSIN. Centrifugation was carried out at 59,780 rpm; bar angle was 60°. In A, cardiac myosin (approximately 3.0 mg per ml) from a normal dog in upper part of picture (wedge cell) and the same preparation with added ATP in the lower picture (regular cell); photograph taken at 52 minutes after reaching maximal speed. In B, two concentrations (1.5 and 1.0 mg per ml) of cardiac myosin from a normal dog after completion of diffusion measurements; picture taken after 72 minutes at a speed of 59,780 rpm. In C and D, cardiac myosin in concentrations of 0.9 and 2.5 mg per ml from a dog with experimental congestive heart failure secondary to tricuspid insufficiency and pulmonic stenosis; photograph in C was taken 12 minutes after reaching maximal speed while picture in D was taken after 68 minutes.

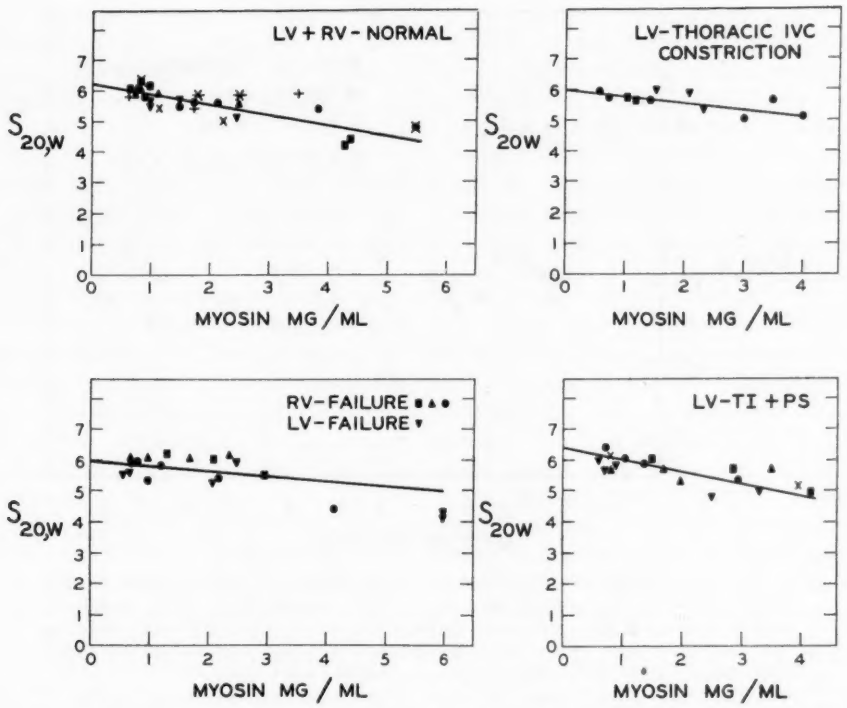

FIG. 2. SEDIMENTATION CONSTANTS ($S_{20,w}$) FOR MYOSIN FROM FOUR DIFFERENT SOURCES PLOTTED AGAINST THE PROTEIN CONCENTRATION. Each symbol represents myosin from a different preparation. Right ventricular failure was secondary to tricuspid insufficiency and pulmonic stenosis. Left ventricular failure was present in the one dog with naturally occurring cardiac failure. RV and LV are abbreviations for right ventricle and left ventricle, respectively. The weighted least squares straight lines are drawn for each group.

significantly greater ($p < 0.01$) than those for myosin from the failing heart.

Diffusion measurements on myosin. Data on the rates of diffusion of myosin from normal cardiac muscle and from cardiac muscle of dogs with cardiac failure are presented in Figure 3. Extrapolation of the $D_{20,w}$ to zero concentration for normal cardiac myosin yields a value of 1.1 $\times 10^{-7}$ cm^2 per second. No differences are apparent in the rates of diffusion of myosin from the normal and experimental material.

Evidence of heterogeneity of the myosin preparations was obtained from analysis of the normalized fringe distances [see Longsworth (16)] at different levels in the diffusing boundary. Maximum deviation of these distances was about 12 per cent, indicating variations of average diffusion coefficients of at least 25 per cent. This indication of polydispersity was apparent in myosin solutions from both normal and experimental hearts.

Viscosity measurements. The viscosity of myosin was measured in enzyme preparations from the combined right and left ventricular masses of normal dogs, from separate right and left ventricles of dogs with right-sided failure secondary to tricuspid insufficiency and pulmonic stenosis, and from the left ventricle only of dogs with thoracic caval constriction. The results are presented in Figure 4 where η_{sp}/C is plotted against the concentration of myosin. For normal cardiac myosin, an excellent linear relation between η_{sp}/C and concentration with evidence of concentration dependence is present between concentrations of 3.5 and 0.4 mg per ml. In the very dilute solutions of myosin (below 0.4 mg per ml), the results varied among different enzyme preparations. With

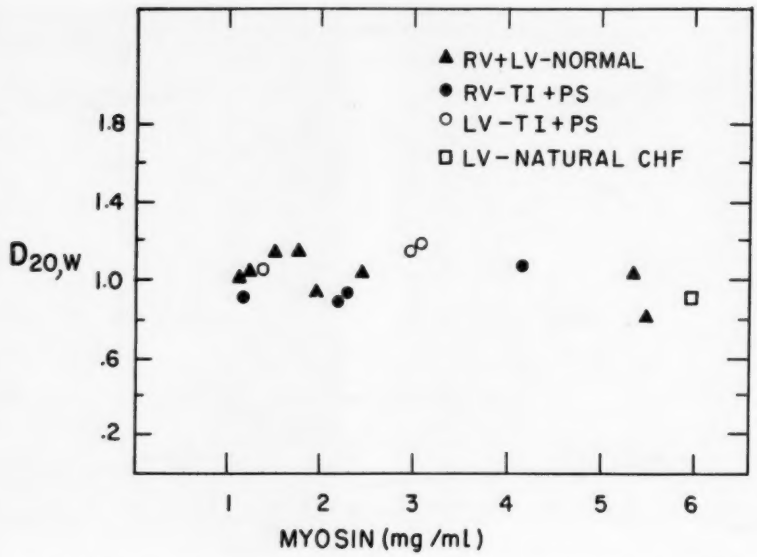

FIG. 3. DIFFUSION COEFFICIENTS ($D_{20,w}$) FOR MYOSIN PLOTTED AGAINST PROTEIN CONCENTRATION. The symbols ● and □ are for myosin from failing ventricular muscle, whereas ▲ and ○ represent myosin from normal muscle and from nonfailing left ventricle of dogs with tricuspid insufficiency (TI) and pulmonic stenosis (PS), respectively. CHF is the abbreviation for congestive heart failure.

one myosin preparation, the η_{sp}/C fell almost to zero, whereas in two other preparations η_{sp}/C increased. If this peculiar behavior at low concentrations is neglected, the intrinsic viscosity (η_{sp}/C at zero concentration) is 1.8. Cardiac myosin from dogs with thoracic caval constriction provided another source of control material (Figure 4); the peculiar viscosity values at very low concentrations were again observed.

The viscosity of myosin from the right ventricle of dogs with right-sided congestive failure did not appear to differ from myosin from normal dogs or dogs with caval constriction. Both myosin from the right ventricle and myosin from the left ventricle of dogs with right heart failure secondary to tricuspid insufficiency and pulmonic stenosis showed an increase in viscosity at low concentrations. Also, one myosin preparation from the left

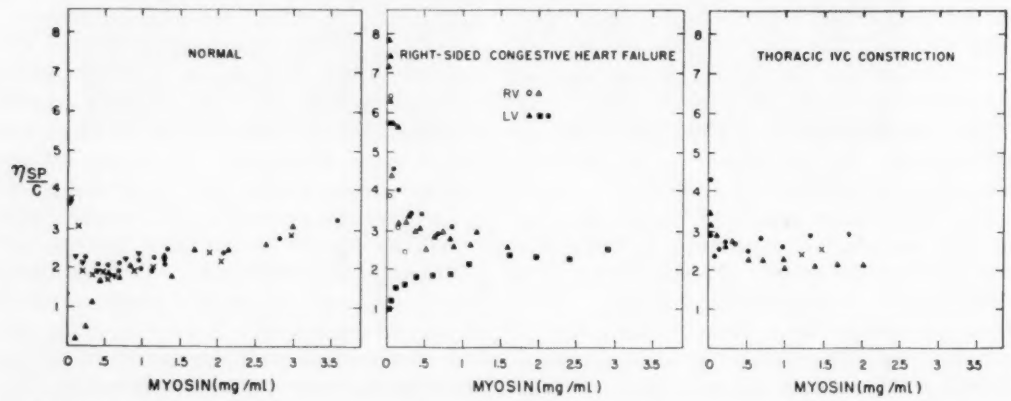

FIG. 4. SPECIFIC VISCOSITY/CONCENTRATION (η_{sp}/C) OF MYOSIN PLOTTED AGAINST THE PROTEIN CONCENTRATION.

ventricle of a dog with right heart failure gave similar response to one myosin preparation from a normal heart in that η_{sp}/C decreased progressively at very low myosin concentrations.

DISCUSSION

The present physicochemical measurements of cardiac myosin have yielded results very similar to those reported for skeletal muscle myosin (1–7). There is good agreement (3–7) that the $S_{20,w}$ for skeletal muscle myosin is in the range of 6.15 to 6.40 $\times$ 10^{-13} seconds. Laki and Carroll (4) obtained a value of 1.0 $\times$ 10^{-7} cm^2 per second for the diffusion coefficient of skeletal myosin. If the peculiar viscosity behavior at concentrations below 0.4 mg per ml is neglected, the present measurements of the intrinsic viscosity of cardiac myosin from normal hearts show a value of 1.8 which is similar to that reported by others (1, 2, 7) for skeletal muscle myosin. Calculations of the molecular weight of cardiac myosin from normal heart muscle, from the present $S_{20,w}$ of 6.17 $\times$ 10^{-13} seconds and the diffusion coefficient of 1.1 $\times$ 10^{-7} cm^2 per second, give a value of 501,000. However, the scatter of sedimentation and diffusion constants is sufficient to include values from 450,000 to 550,000 for the molecular weight.

Also, the findings in this study are in excellent agreement with the results of Gergely and Kohler (9) from studies of normal cardiac myosin. They obtained a value of 500,000 for the molecular weight from light scattering measurements. The present data and those of Gergely and Kohler appear to be at variance with the finding of Olson (8) of a value of 223,000 for normal cardiac myosin. However, the results from all three laboratories would be reconcilable if Olson's myosin preparations consisted of monomers, whereas the cardiac myosin prepared by Gergely and Kohler and the present preparations consisted mostly of myosin in the form of dimers.

The close similarity of the magnitude of the parameters from the present data on cardiac myosin to those of Holtzer and Lowey (7) and others (2–6) for skeletal myosin suggests that the shape of the cardiac myosin molecule is very similar, if not identical, to that for skeletal myosin. From sedimentation, viscosity and light scattering measurements, Holtzer and Lowey (7) reported a molecular weight of 493,000 for skeletal muscle myosin. They suggested that myosin is a rod 1,620 Å long and 26 Å thick. If myosin preparations which yield values of approximately 500,000 for the molecular weight consist mostly of dimers, it appears that the two monomers of myosin are arranged end-to-end to form a dimer. In this event, the molecular dimensions described by Holtzer and Lowey (7) would apply to the dimer.

Another similarity between the present cardiac myosin preparation and skeletal muscle myosin is the occurrence of polydispersity. The diffusion data in this study provide evidence for a polydisperse system from analysis of the normalized fringe distances at different levels in the diffusing boundary. In the ultracentrifuge patterns, the occasional presence of a small sedimentation component which moved faster than the principal sedimenting boundary is probably a reflection of polymerized myosin. Aggregation of the basic myosin monomer, with the resultant appearance of a fast-moving component during ultracentrifugation, has been observed for skeletal muscle myosin (7, 17).

The present viscosity measurements of cardiac myosin showed a peculiar finding in very dilute solutions (below 0.5 mg per ml). The η_{sp}/C increased markedly in some preparations and decreased in others. The phenomenon occurred with myosin from both normal and failing hearts and with cardiac myosin from dogs with thoracic caval constriction. Similar changes in the viscosity of cardiac myosin at low concentrations have been reported previously by Olson (8). In view of the the irreproducibility of the viscosity measurements among different myosin preparations from the same sources of material (normal or failing ventricle), the possibility must be considered that viscosity measurements at very low concentrations do not represent the true viscosity of the solution. It does not seem likely that the viscosity changes at low concentrations were the result of the temperature (22.5° C) at which viscosity was measured, since Olson (8) observed the same peculiar viscosity at 1° C. Also, Holtzer and Lowey (7) concluded that the slight difference in intrinsic viscosity of myosin measured at 1° and at 26° C was probably not significant.

The results of this study show no difference between cardiac myosin from normal dog hearts and from either the right or left ventricle of dogs

with right-sided congestive failure secondary to tricuspid insufficiency and pulmonic stenosis. Also, left ventricular myosin from the one dog with naturally occurring right and left ventricular failure appeared normal. These findings are in contrast to the results of Olson (8) who reported that an abnormal cardiac myosin was extracted from the combined right and left ventricles of dogs with tricuspid insufficiency and pulmonic stenosis. Olson and Piatnek (18) reported that their dogs with tricuspid insufficiency and pulmonic stenosis were in left ventricular failure.

In the present study, cardiac myosin was obtained from separate right and left ventricles of the animals with cardiac failure. This procedure was adopted so that myosin from the specific failing chamber (right or left ventricle) could be compared with normal material. There was no evidence for left ventricular failure in the present dogs with tricuspid insufficiency and pulmonic stenosis, and an earlier more extensive study (13) also revealed pure right-sided cardiac failure in dogs with tricuspid insufficiency and pulmonic stenosis.

It has been suggested by Benson (19) that actomyosin is partially fragmented into uncombined myosin and actin in dogs with experimental cardiac failure secondary to tricuspid insufficiency and pulmonic stenosis. However, recent extensive studies of cardiac actomyosin (10) have failed to confirm the findings of Benson. Sedimentation velocity and viscosity measurements of cardiac actomyosin from the right ventricle of dogs with chronic experimental congestive failure, produced by either tricuspid insufficiency and pulmonic stenosis or by severe pulmonic stenosis alone, showed no evidence for altered actomyosin in 7 of 11 dogs. In the other four animals, a slow sedimenting component was present in the sedimentation diagrams but this appeared to be a preparation or extraction artifact. The present observations and those reported previously on actomyosin (10) offer no support for the view that altered contractile proteins constitute the biochemical defect in the failing heart.

SUMMARY AND CONCLUSIONS

The molecular weight of cardiac myosin was determined from sedimentation and free diffusion data; a value of approximately 500,000 was obtained for normal cardiac myosin. Measurements of the intrinsic viscosity of cardiac myosin from normal hearts showed a value of 1.8. Sedimentation velocity, viscosity and diffusion measurements of myosin showed no differences between myosin prepared from normal hearts and myosin obtained from failing ventricular muscle. Also, the present physicochemical constants for cardiac myosin are in good agreement with data described by others for skeletal muscle myosin.

ACKNOWLEDGMENT

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MECHANISMS OF IMPAIRED WATER EXCRETION IN ADRENAL AND PITUITARY INSUFFICIENCY. II. INTERRELATIONSHIPS OF ADRENAL CORTICAL STEROIDS AND ANTIDIURETIC HORMONE IN NORMAL SUBJECTS AND IN DIABETES INSIPIDUS *

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The impaired water diuresis of adrenal or pituitary insufficiency is completely corrected by the administration of cortisol and other glucocorticoid analogues (1–5). This correction results not from improved renal hemodynamics, altered tubular reabsorption of solutes, or removal of a nonosmotic (volume) stimulus to continued antidiuretic hormone (ADH) release, but probably from a direct effect of the steroid on the diluting segments of the nephrons (*2*, 6). This interpretation implies that the formation, release and inactivation of ADH are normal in the adrenal-insufficient subject. Most of the available data in humans would tend to confirm this implication (1, 2, 5–14). Others, however, have suggested that glucocorticoids may be either biological antagonists of ADH (15–19) or may improve the impaired water excretion in adrenal and pituitary insufficiency by correcting a defect in the metabolism of ADH (18, 20–22).

In the present investigation, the interrelationship between adrenal glucocorticoids and ADH was evaluated by observing the effect of the steroid on the parameters of water diuresis and antidiuresis. The experiments were designed to answer the following questions. What is the effect of acute and chronic administration of glucocorticoid on: *1*) maximal sustained water diuresis in normals and in patients with diabetes insipidus? *2*) the acute and sustained release of ADH from the neurohypophysis following both osmotic and nonosmotic stimuli? *3*) the rate of inactivation of circulating ADH following inhibition of its release from the neurohypophysis? and *4*) the renal action of endogenous and exogenous ADH? The results indicate that these steroids do not alter the release, inactivation, or action of ADH in normal individuals.

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METHODS AND MATERIALS

Forty-three experiments were performed on 20 subjects. Sixteen were normal house staff physicians and laboratory technicians, two were patients in the euthyroid phase following I^{131} therapy of thyrotoxicosis, and two were well documented cases of idiopathic diabetes insipidus. All subjects were between the ages of 20 and 40. Each subject served as his own control in paired studies of the effects of exogenous glucocorticoids on water metabolism. The steroids given were cortisol, 150 to 200 mg, or 6-methyl-prednisolone (Medrol), 20 mg. Chronic administration consisted of giving the steroid orally for 4 to 5 days prior to and, in an intravenous drip, during the experiment. "Acute" administration consisted of giving the steroid during the experiment only. In some paired studies the steroid was given during the first study; in others the procedure was reversed.

The technique employed for attaining a maximal sustained water diuresis, the calculation of the parameters of diuresis and antidiuresis, and the chemical procedures used in this laboratory have been described previously (*2*). When the steroid was administered on the day of an experiment, it was given as a constant intravenous infusion of 20 mg of 6-methyl-prednisolone (3 mg per hour) or 100 mg of cortisol (15 to 20 mg per hour) throughout the experiment.

RESULTS

The effect of acute and chronic steroid administration on the parameters of maximal sustained water diuresis in normal subjects and in patients with diabetes insipidus (Table I)

When the adrenal steroid was given acutely, the control periods of maximal diuresis were com-

TABLE I

Effect of acute and chronic steroid administration on maximal sustained water diuresis *

Subject	Date	Exp.	Urine vol.	Urine conc.	Solute excr.	Free water clearance (C_{H_2O})	Inulin clearance (GFR)	PAH clearance (RPF)	Serum osmol.
			cc/min	*mOsm/kg*	*μOsm/min*	*cc/min*	*cc/min*	*cc/min*	*mOsm/kg*
			Chronic administration (normal subjects)						
1	9/11/58	A†	12.6	67	843	9.5	112	519	274
	9/18/58	B‡	18.4	69	1,266	13.9	110	520	280
2	12/14/57	B	29.4	78	2,293	21.1	173	738	275
	12/26/57	A	23.2	51	1,256	18.7	167	762	276
3	2/7/58	A	22.2	76	1,687	16.2	105		278
	2/21/58	B	22.4	48	850	19.2	100		275
4	4/21/59	B§	20.5	65	1,334	15.7	155	596	277
	4/28/59	A§	19.3	77	1,488	13.9	137	560	276
	5/15/59	B	20.5	44	902	17.2	129	586	271
5	11/21/58	A	13.2	56	739	10.6	90		279
	11/26/58	B	17.0	53	901	13.8	116		282
6	1/12/59	A	19.1	76	1,451	14.0	180	645	285
	1/16/59	B	22.2	60	1,330	17.5	164	695	286
7	2/10/59	A	11.7	78	913	8.5	125	500	282
	2/15/59	B	14.1	74	1,043	10.4	131	642	286
8	3/27/59	B§	21.1	75	1,586	15.7	134	579	290
	4/10/59	A	14.9	61	907	11.7	107	470	289
9	3/13/59	A	14.8	90	1,328	10.2	100	557	290
	3/18/59	B	18.0	72	1,296	13.7	138	565	295
10	8/1/59	B	19.1	41	783	16.4	150	816	291
	8/7/59	A	15.0	44	660	12.7	135	780	291
11	7/7/59	A	13.2	86	1,133	9.8	134	818	285
	7/16/59	B	18.8	52	980	15.3	134	880	275
	7/25/59	B	18.6	41	763	16.0	137	838	288
	9/20/59	A	18.7	58	1,085	14.7	144	881	270
12	7/17/59	B	18.0	61	1,098	14.0	138	799	272
	7/23/59	A	16.8	61	1,027	13.1	173	814	273
			Diabetes insipidus patients						
13	10/7/58	A	16.1	60	960	12.4	110	500	270
	10/12/58	B	24.5	42	1,029	21.0	120	580	287
14	10/31/58	A	16.2	49	795	13.2	160	740	260
	11/5/58	B	30.8	70	2,157	23.0	225	960	277
			Acute administration						
15	12/28/57	A	16.5	38	627	14.3	104		275
	12/28/57	B	18.2	50	910	14.8	100		280
16	11/19/57	A	15.8	88	1,390	10.8	148		280
	11/19/57	B	16.8	88	1,478	11.5	153		280
17	11/5/57	A	17.1	51	872	14.1	137		294
	11/5/57	B	17.5	54	945	14.3	142		295
18	4/15/57	A	16.8	62	1,042	13.3	228	733	298
	4/15/57	B	15.6	61	952	12.3	206	660	292

* All values represent the mean of two or three clearance periods at the height of the diuresis.
† A refers to control experiments (or periods in the acute studies) without steroid administration.
‡ B refers to experiments (or periods) following acute or chronic steroid administration.
§ These subjects received 6-methyl-prednisolone rather than cortisol.

pared with those periods during the 4 hours of continuous steroid infusion. In no instance did an augmentation of the maximal diuresis occur (Subjects 15–18). In contrast, following chronic administration of the steroid, 12 of the 14 subjects demonstrated a significant enhancement of the water diuresis, as measured by all parameters (Subjects 1, 2, 5, 7–11, 13 and 14) or by free water clearance (C_{H_2O}), and minimal osmolality (Subjects 3, 6, and 11–2 [1]). The water diuresis in Subject 4 was not enhanced with 6-methyl-prednisolone, but it was with cortisol. In six subjects (Subjects 2, 5, 8–10 and 14), the increase in renal hemodynamics and/or solute excretion

[1] The "2" represents the second paired experiment done on this patient.

was of such a magnitude as to contribute significantly to the augmented diuresis. While it is unlikely that the magnitude of the augmentation of the water diuresis in Subject 1 can be explained by the 50 per cent increase in solute excretion, the contribution of the latter cannot be ignored. In the remaining experiments (Subjects 3, 6, 7, 11–1, 11–2 and 13), the increment in free water clearance could not be explained by enhanced renal hemodynamics or solute excretion. The observations in Table I indicate that maximal water diuresis can be enhanced by the chronic administration of glucocorticoids in subjects with clinical as well as physiological diabetes insipidus (maximal sustained water load).

The effect of steroid administration on the acute and chronic release of ADH from the neurohypophysis following osmotic and nonosmotic stimuli (Table II and Figures 1 and 2)

A. Osmotic stimuli. When 400 ml of 5 per cent hypertonic saline was infused into two subjects during a maximal sustained water diuresis, the time of onset and the magnitude of the antidiuresis were not altered by chronic administration of cortisol (Subjects 19 and 20). The pattern of antidiuresis in a paired study is illustrated in Figure 1.

A chronic osmotic stimulus to the release of ADH was produced in two subjects by 24 hours of total restriction of fluid intake and the ingestion of a liquid-free diet. Chronic steroid administration did not lower the maximal urinary osmolality or the magnitude of the negative free water clearance ($T^c_{H_2O}$) following this period of hydropenia (Subjects 21 and 22).

B. Nonosmotic stimuli (Table III and Figures 2 and 3). The results of this group of experiments were difficult to interpret because of the variability of response of the subjects to the nonosmotic stimuli.

1. Inhalation of cigarette smoke during a maximal water diuresis. Nicotine is a strong stimulus to the release of antidiuretic hormone (23) and inhalation of cigarette smoke is a relatively reliable substitute for the parenteral administration of

TABLE II

The effect of chronic adrenal steroid administration on the acute and chronic release of ADH following an osmotic stimulus *

Subject	Date	Exp.	Urine vol.	Urine conc.	Solute excr.	Free water clearance (C_{H_2O})	Inulin clearance (GFR)	PAH clearance (RPF)	Serum osmol.
			cc/min	*mOsm/kg*	*μOsm/min*	*cc/min*	*cc/min*	*cc/min*	*mOsm/kg*
		Hypertonic saline during a maximal water diuresis							
19	12/14/57	B† Control	29.4	78	2,293	21.1	173	736	275
	12/14/57	Hypertonic salt‡	15.0	290	4,350	−0.8	217	878	275
	12/26/57	A§ Control	23.2	51	1,256	18.7	167	762	276
	12/26/57	Hypertonic salt‡	12.0	316	3,792	−1.4	178	800	283
20	7/17/59	B Control	18.0	61	1,098	14.0	138	799	272
	7/17/59	Hypertonic salt‡	1.6	706	1,165	−2.4	164	646	288
	7/24/59	A Control	16.8	61	1,027	13.1	173	814	273
	7/24/59	Hypertonic salt‡	3.0	593	1,791	−3.4	168	754	279
		Maximal antidiuresis following 24 hours of dehydration							
21	9/26/58	A	0.6	849	509	−1.3	150	600	270
	10/2/58	B	0.6	875	525	−1.3	143	562	270
22	1/13/57	A	0.6	1,055	634	−1.5	213		300
	1/15/57	B	0.5	1,065	532	−1.3	212		295

* All values represent the mean of two or three clearance periods at the height of the diuresis and the values from the period of maximal antidiuresis.

† B refers to experiments (or periods) following acute or chronic steroid administration.

‡ 400 ml of 5 per cent saline.

§ A refers to control experiments (or periods in the acute studies) without steroid administration.

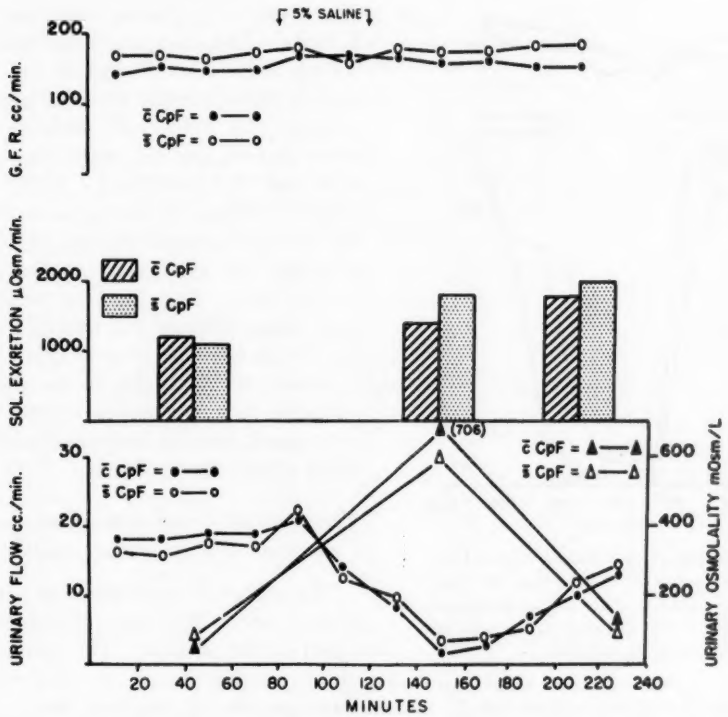

FIG. 1. THE EFFECT OF CORTISOL ADMINISTRATION ON THE ANTIDIURETIC RESPONSE TO AN INFUSION OF 5 PER CENT SALINE DURING A MAXIMAL SUSTAINED WATER DIURESIS (SUBJECT 20). The triangles refer to the osmolality of the urine. The figures in parentheses represent the maximal urinary osmolality.

nicotine tartrate or the pure alkaloid (23).[2] At the height of the sustained water diuresis, therefore, the subjects smoked and inhaled completely four cigarettes over a 10 to 20 minute period. The effect of smoking was subsequently observed for 2.5 hours. In Subjects 23 and 24, smoking caused a pronounced antidiuresis and significant hypertonicity of the urine. The magnitude and pattern of the antidiuresis and the pattern of return to maximal flow of urine was not altered by chronic administration of steroid. This is illustrated in Figure 2 for Subject 24. The marked antidiuretic response to nicotine observed in Subject 25 was not reproduced after 4 days of steroid ingestion. This difference could not be explained by altered renal hemodynamics or solute excretion. Nicotine inhalation without steroid administration caused only a moderate antidiuresis in Subject 26. This antidiuresis was due in part to a significant decrease in solute excretion, renal plasma flow (RPF), and glomerular filtration rate (GFR). When nicotine inhalation was repeated after chronic steroid administration, the antidiuresis was partially (6-methyl-prednisolone 4/21/59) or completely (cortisol 5/15/59) blocked. However, renal hemodynamics and solute excretion changed very little in the experiments done under the influence of the adrenal steroids.

The moderate antidiuresis caused by cigarette smoking in Subject 27 was not altered by chronic steroid administration.

2. Venous congestion of the lower extremities. Another nonosmotic stimulus to the release of ADH (24) was achieved by producing venous

[2] Although it was realized that the injection of nicotine would give the most consistent results, initial trials with parenteral administration produced such severe side reactions that it was considered inadvisable to continue this mode of administration.

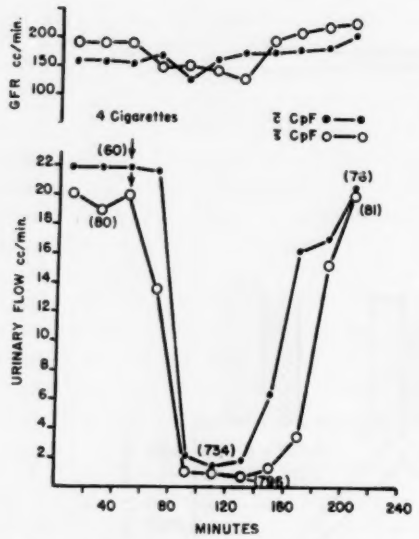

FIG. 2. THE EFFECT OF CORTISOL ADMINISTRATION ON THE ANTIDIURETIC RESPONSE TO CIGARETTE SMOKING DURING A MAXIMAL SUSTAINED WATER DIURESIS. The figures in parentheses represent the urinary osmolality.

obstruction of the lower extremities for 40 minutes by placing sphygmomanometer cuffs high on both thighs. Venous congestion was initiated after a sustained maximal diuresis had been achieved. The effects of congestion were observed for 2.5 to 3 hours. The moderate antidiuresis induced by cuffing in Subjects 28 and 29 was unaffected by chronic steroid administration (Figure 3). Venous congestion in Subject 30 markedly inhibited the water diuresis, and it appeared that administration of steroid on 7/16/59 and 7/25/59 "blocked" the effect of cuffing. However, when the experiment was repeated *without steroids* (9/20/59), venous congestion did not cause a significant antidiuresis (Table III). The effect of venous congestion upon water diuresis was thus not clearly altered by chronic administration of glucocorticoid.

Despite the variability in the release of ADH following these nonosmotic stimuli, the results do not suggest that the steroids significantly or regularly altered the responses.

The effect of steroid administration on the rate of inactivation of circulating ADH

The pattern of development of a maximal water diuresis and its subsequent disappearance was followed in two subjects. The intravenous infusion of 2.5 per cent glucose was stopped after two or three periods of maximal flow were obtained. Both subjects showed a comparable pattern of response. This is illustrated in Figure 4 for one of the subjects. It is evident that chronic steroid

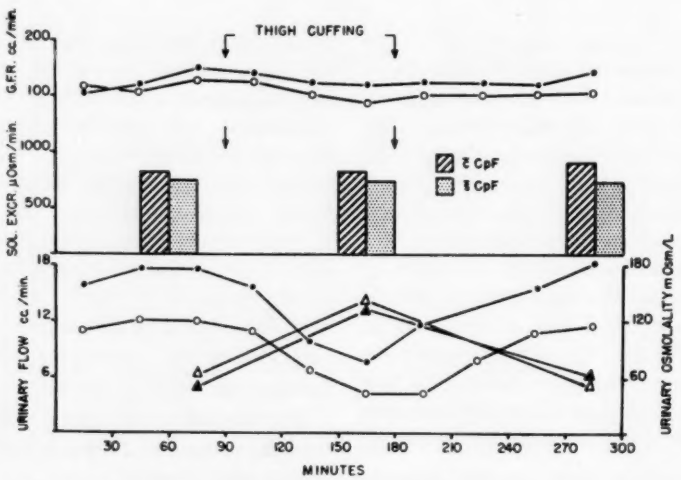

FIG. 3. THE EFFECT OF CORTISOL ADMINISTRATION ON THE ANTIDIURETIC RESPONSE TO VENOUS CONGESTION OF THE LOWER EXTREMITIES DURING A MAXIMAL SUSTAINED WATER DIURESIS. The open circles and triangles represent the control study; the closed, the study with steroid administration.

TABLE III

The effect of chronic adrenal steroid administration on the acute release of ADH following a nonosmotic stimulus *

Subject	Date	Exp.	Urine vol.	Urine conc.	Solute excr.	Free water clearance (C_{H_2O})	Inulin clearance (GFR)	PAH clearance (RPF)	Serum osmol.
			cc/min	*mOsm/kg*	*μOsm/min*	*cc/min*	*cc/min*	*cc/min*	*mOsm/kg*
		Cigarette smoking							
23	3/27/59	B† Control	21.1	75	1,586	15.7	134	579	290
	3/27/59	Nicotine	1.8	580	1,050	−1.9	148	599	278
	4/10/59	A‡ Control	14.9	61	907	11.7	107	470	289
	4/10/59	Nicotine	1.4	700	987	−2.1	108	481	279
24	1/12/59	A Control	19.1	76	1,451	14.0	180	645	285
	1/12/59	Nicotine	1.2	796	946	−2.2	175	672	283
	1/16/59	B Control	22.2	60	1,330	17.5	164	695	286
	1/16/59	Nicotine	1.2	734	888	−1.8	158	603	289
25	3/13/59	A Control	14.8	90	1,328	10.2	100	557	290
	3/13/59	Nicotine	1.3	589	760	−1.4	102	455	289
	3/18/59	B Control	18.0	72	1,296	13.7	138	565	295
	3/18/59	Nicotine	8.1	122	988	4.7	138	407	287
26	4/21/59	B§ Control	20.5	65	1,334	15.7	155	596	277
	4/21/59	Nicotine	9.6	101	975	6.1	141	507	275
	4/28/59	A Control	19.3	77	1,488	13.9	137	560	276
	4/28/59	Nicotine	4.2	156	645	1.9	116	445	274
	5/15/59	B Control	20.5	44	902	17.2	129	586	271
	5/15/59	Nicotine	19.6	47	921	16.3	126	531	273
27	2/10/59	A Control	11.7	78	913	8.5	125	500	282
	2/10/59	Nicotine	6.9	142	970	3.4	122	523	282
	2/15/59	B Control	14.1	74	1,043	10.4	131	642	286
	2/15/59	Nicotine	6.8	168	1,142	2.8	136	658	284
		Venous congestion of lower extremities							
28	9/11/58	A Control	12.6	67	843	9.5	112	519	274
	9/11/58	Cuffing	4.6	123	563	2.5	100	400	279
	9/18/58	B Control	18.4	69	1,266	13.9	110	520	280
	9/18/58	Cuffing	8.5	108	927	5.4	137	770	296
29	8/1/59	B Control	19.1	41	783	16.4	150	816	291
	8/1/59	Cuffing	4.6	113	523	2.9	123	653	296
	8/7/59	A Control	15.0	44	660	12.7	135	780	291
	8/7/59	Cuffing	7.4	56	414	6.0	130	690	291
30	7/7/59	A Control	13.2	86	1,133	9.8	134	818	285
	7/7/59	Cuffing	1.8	416	761	−0.8	155	778	285
	7/16/59	B Control	18.8	52	980	15.3	134	880	275
	7/16/59	Cuffing	11.9	61	726	9.6	135	773	285
	7/25/59	B Control	18.6	41	763	16.0	137	838	288
	7/25/59	Cuffing	15.4	42	645	13.1	135	885	288
	9/20/59	A Control	18.7	58	1,085	14.7	144	881	270
	9/20/59	Cuffing	15.8	60	948	12.3	151	770	270

* All values represent the mean of two or three clearance periods at the height of the diuresis and the values from the period of maximal antidiuresis.
† B refers to experiments (or periods) following acute or chronic steroid administration.
‡ A refers to control experiments (or period in the acute studies) without steroid administration.
§ This subject received 6-methyl-prednisolone rather than cortisol.

administration did not alter the characteristics of the water diuresis curve.

Two subjects received an infusion of the same lot of Pitressin (vasopressin, 200 mU per hour) at the height of the water diuresis. When maximal antidiuresis was achieved, the infusion of vasopressin was stopped and the return toward a maximal diuresis was observed. Both subjects showed a comparable response. This is illustrated for one subject in Figure 5. Chronic steroid administration did not alter the pattern of response to exogenous vasopressin. Similarly, the curve of urinary flow following release of endogenous ADH was not affected by the steroids (Figure 2). It is apparent from these experiments that the rate of "inactivation" of exogenous or endogenous ADH was not modified by prior ingestion of adrenocortical steroids.

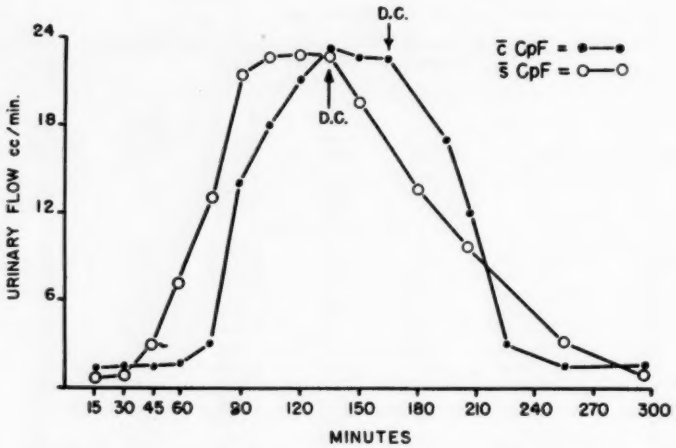

FIG. 4. THE EFFECT OF CORTISOL ADMINISTRATION ON THE PATTERN OF DEVELOPMENT AND DISAPPEARANCE OF A MAXIMAL WATER DIURESIS. "D.C." indicates the time when the sustained water infusion was discontinued.

The effect of steroid administration on the renal responsiveness to endogenous or exogenous ADH

If the glucocorticoids directly inhibited the action of antidiuretic hormone on the renal tubule, their administration should have caused a consistent reduction in the maximal antidiuretic response to endogenous or exogenous ADH. This was not observed in any of the experiments. However, it must be stated that the experiments as performed do not indicate whether the *minimal* dose of ADH necessary to produce an antidiuretic response is altered by steroid administration.

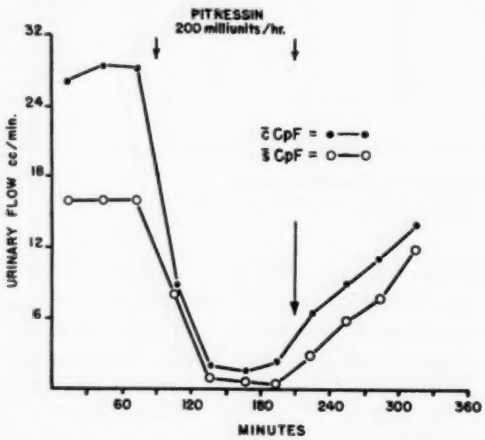

FIG. 5. THE EFFECT OF CORTISOL ADMINISTRATION ON THE ANTIDIURETIC RESPONSE TO AN INTRAVENOUS INFUSION OF VASOPRESSIN (200 MU PER HOUR).

DISCUSSION

A physiological "antagonism" or interaction between the glucocorticoids of the adrenal cortex and the neurohypophyseal system and its hormone (ADH) has frequently been proposed (15–22); namely, excessive amounts of adrenal steroids appear to block the release of ADH from the neurohypophysis (16–18, 20), while deficiency of these cortical hormones enhances its release (19, 20, 22), delays its inactivation (19, 21), or increases the renal tubular sensitivity to its action (20). The majority of these studies have been performed in normal or adrenalectomized rats (19–21) and for the most part have not been confirmed in humans with primary or secondary adrenal insufficiency (2, 5, 7, 10, 11, 13, 14).

In the present study an attempt was made to demonstrate in normal subjects an interaction between glucocorticoids and the metabolism of ADH at three levels. These were: *1*) the release of ADH from the neurohypophysis; *2*) the inactivation of circulating ADH; and *3*) its action on the renal tubule. As part of this study, it seemed essential to demonstrate whether adrenal steroids could alter maximal water diuresis in the absence of circulating ADH (physiological or clinical diabetes insipidus). It was shown that this aug-

mentation could occur without a change in renal hemodynamics or solute excretion, thus confirming the observations of Raisz, McNeely, Saxon and Rosenbaum (9) and those of Vesin (25). These results should be supplemented by those showing that adrenal steroids can markedly improve the impaired water diuresis in patients (7–12, 14) and in animals (26) with combined anterior and posterior pituitary insufficiency. One can then conclude that augmentation of water diuresis in the above circumstances certainly is not due to an alteration of the metabolism of ADH.

Because of the variability in response to venous congestion and inhalation of cigarette smoke, these results were not as conclusive as the others. However, they did not indicate a consistent effect of the glucocorticoids on the nonosmotic release of ADH. It is reasonable to conclude that chronic steroid administration to the normal subjects of the present study did not modify the secretion (release), "inactivation," or action of antidiuretic hormone.[3]

SUMMARY AND CONCLUSIONS

1. The interrelationships of adrenal steroids and antidiuretic hormone (ADH) were evaluated in normal subjects.

2. Chronic steroid administration significantly augmented the maximal water diuresis of normal subjects and patients with diabetes insipidus. Although this augmentation was frequently accompanied by increased renal hemodynamics and/or solute excretion, it may occur without a significant change in these parameters.

3. No evidence was obtained to indicate that the release, inactivation, or renal tubular action of ADH was affected by prior steroid administration.

4. In normal human subjects, an antagonism between ADH and adrenal glucocorticoids could not be demonstrated.

[3] The results of the present study are somewhat at variance with those of Dingman, Thorn and Despointes (16–18). They stated that acute or chronic steroid administration raised the threshold of response of the neurohypophysis to the nonosmotic stimulus, nicotine, and that the correction of the impaired water metabolism in adrenal insufficiency and the augmentation of water diuresis in the normal subject was due to an effect of adrenal steroids on the neurohypophyseal system. We are unable to evaluate their findings, since two of the reports appeared only in abstract form (16, 17) and one was a review article without original data (18).

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LEUKOKINETIC STUDIES. II. A METHOD FOR LABELING GRANULOCYTES *IN VITRO* WITH RADIOACTIVE DIISOPROPYLFLUOROPHOSPHATE (DFP^{32})*

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In a previous publication from this laboratory (1), radioactive diisopropylfluorophosphate (DFP^{32}), a potent and irreversible esterase inhibitor, was shown to be a suitable label for granulocytes. Evidence was presented that the label is firmly attached to granulocytes; it does not modify the viability of the cells, and the label is not reutilized when the cells are degraded. Experiments designed to determine whether or not elution of the label occurs failed to provide such evidence. More recent studies (2), carried out with tritiated DFP and autoradiography, have confirmed the observation that the label is attached to granulocytes and little, or not at all, to lymphocytes or to monocytes.

A method for labeling granulocytes *in vivo* with DFP^{32} was described in the previous publication (1). The purpose of this paper is to describe a method for labeling granulocytes *in vitro*, and to describe the manner in which granulocytes so labeled leave the circulation of normal subjects. Several preliminary reports of this work have been published (3, 4).

MATERIAL AND METHODS

Subjects. A total of 82 volunteers from the Utah State Prison was used in these studies. All of the subjects were normal, healthy males, 20 to 50 years of age, with normal leukocyte values. Each subject was used only once in the study. All of the studies were started between 8 and 10 a.m.

Method of labeling granulocytes in vitro. Five hundred ml of blood was withdrawn from the subject into a sterile, plastic bag [1] containing 67.5 ml of ACD-A solution.[2] DFP^{32} was added to the blood in the bag in a concentration of 0.3 μg per ml of whole blood and the contents were mixed gently. The blood was allowed to remain at room temperature for 1 hour with occasional gentle mixing. At the end of this time, a 20 ml sample of blood was obtained from the bag and the remainder of the blood was infused into the subject over a 10 to 15 minute period.

Samples of blood, 20 ml each, were withdrawn at 0, 3, 6, 10, 24 and 30 hours after completion of the infusion. The methods for the isolation of the leukocytes from the blood samples and for determination of leukocyte radioactivity have been described previously (1).

Calculations. The percentage of cells infused which could be demonstrated in the circulation immediately after the infusion was calculated in the following manner. The specific activity (counts per minute per milligram leukocyte nitrogen) of the leukocytes in the bag was determined at the time of the infusion. From this determination, the volume of blood injected, and the calculated blood volume (5, 6), the expected counts per minute of the leukocytes in the circulation immediately after completion of the 15 minute infusion (t_0) were calculated:

$$\text{expected specific activity cells in blood at } t_0 = \frac{\text{specific activity of cells in bag} \times \text{volume of blood labeled}}{\text{blood volume}}$$

The specific activity of the cells in the blood immediately after the infusion (t_0) was determined by extrapolation of the exponential granulocyte disappearance curve to the ordinate. The percentage of infused cells which was present in the circulation at t_0 was then calculated as follows:

$$\frac{\text{determined specific activity of cells at } t_0}{\text{expected specific activity of cells at } t_0} \times 100 = \text{percentage of infused cells present in the circulation at } t_0.$$

The time for half of the labeled granulocytes to leave the blood ($T_{\frac{1}{2}}$) was determined by plotting the leukocyte radioactivities against time in hours on semilogarithmic paper.

Radiation dosage to the subjects. When DFP^{32} with a specific activity of 200 μc per mg was used, a total of 30 μc of radioactivity was administered to each subject. The

* This investigation was supported in part by research grants (C-2231 and CY-2349) from the National Cancer Institute and in part by a graduate training grant (2A-5098) from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

† Aided by a Postdoctoral Fellowship from the American Cancer Society.

[1] Pliapak, Abbott Laboratories, North Chicago, Ill.

[2] Each 100 ml contains 2.45 g of dextrose, 2.20 g of sodium citrate, and 0.73 g of citric acid.

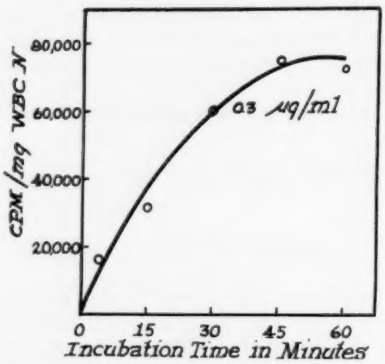

FIG. 1. UPTAKE OF DFP^{32} BY GRANULOCYTES IN RELATION TO TIME.

compound was presumably bound firmly to erythrocytes, leukocytes, platelets and plasma proteins and, therefore, 30 μc of isotope was distributed predominantly in about 5 kg of blood with very little radioactivity reaching the bone marrow or other tissues. In three subjects, in whom the urinary excretion of radioactivity was studied, 40 per cent of the administered radioactivity appeared in the urine within the first 8 hours after infusion of the labeled blood. Thereafter, the biological half-life of the radiophosphorus in the body was approximately 6 days. The effective half-life was calculated to be 4.4 days (7). With the nomogram of Bertinchamps and Cotzias (7), the infinite dose of radioactivity administered to blood was calculated to be about 860 millirads. This figure represents the maximum dose, since the above calculation of the biological half-life does not take into consideration the excretion of DFP^{32} through routes other than the kidneys.

Analysis of the DFP^{32} preparations. DFP^{32} was purchased from a commercial source [3] as a dilute solution in sterile, anhydrous propylene glycol. The amount of DFP^{32} present in the solution was determined by the colorimetric method of Marsh and Neale (8) as modified by Wilcox and Neurath.[4]

Nine different batches of DFP^{32} were used in these studies. The determined DFP concentrations were found to range from 15 to 100 per cent of the concentration stated by the manufacturer. The specific activities of the batches, at the time the material was used, ranged from 103 to 336 μc per mg.

RESULTS

Rate of uptake of DFP^{32} into leukocytes. To determine the period of incubation required to obtain optimal labeling of granulocytes, blood was drawn into a plastic bag and incubated for 1 hour at room temperature with 0.3 μg of DFP^{32} per ml of whole blood. The uptake of DFP^{32} into the leukocytes was maximal in about 45 minutes (Figure 1).

The following experiment was performed to determine whether or not any DFP^{32}, which would be capable of labeling the leukocytes of the recipient, remained in the plasma after a 1 hour incubation period. From each of three subjects, 500 ml of blood was withdrawn. The blood was incubated at room temperature for 1 hour with 0.3 μg of DFP^{32} per ml. At the end of this time, the blood was centrifuged to remove all cells and the cell-free plasma was infused into the donors. No radioactivity could be demonstrated subsequently in either the leukocytes or the erythrocytes of the subjects.

Disappearance of labeled granulocytes from the circulation. The disappearance of labeled granulocytes from the blood was measured in 45 normal subjects. The labeled leukocytes left the circulation in an exponential fashion in all subjects. The leukocyte radioactivity disappearance curves obtained in four representative subjects are shown in Figure 2.

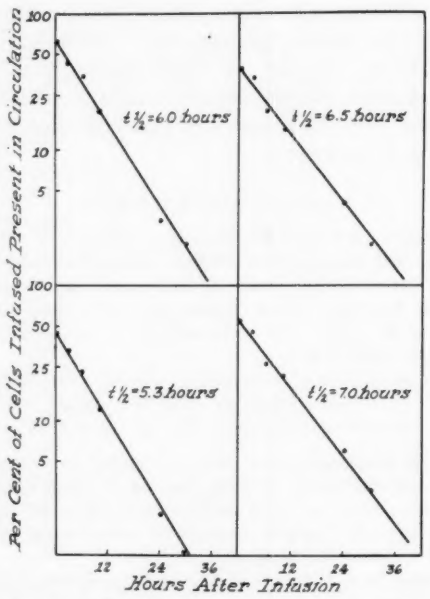

FIG. 2. GRANULOCYTE RADIOACTIVITY CURVES IN FOUR REPRESENTATIVE SUBJECTS.

[3] Manning Research Laboratories, Waltham, Mass.

[4] We are indebted to Dr. Hans Neurath, University of Washington, Seattle, Wash., for supplying us with this method.

TABLE I

Influence of DFP^{32} concentration *

Concentration	No. of subjects	Percentage of infused cells present at t_0 Mean	Range	$T_{\frac{1}{2}}$ Mean	Range
μg/ml		%	%	*hrs*	*hrs*
0.1	4	33	27–39	7.0	6.0–8.0
0.2	3	42	26–68	7.5	6.0–9.0
0.3	8	49	27–62	6.9	5.5–8.0
0.7	3	23	14–33	7.2	5.8–8.5
1.0	3	42	29–57	8.3	7.0–9.0

* Five hundred ml of blood was incubated in a plastic bag with ACD-A for 60 minutes at room temperature with the concentrations of DFP^{32} given above.

The mean $T_{\frac{1}{2}}$ ($\pm$ one standard deviation) in the circulation was 6.6 $\pm$ 1.4 hours in the group of 45 normal male subjects, with a range of from 4.0 to 9.0 hours.

The percentage of infused cells which could be demonstrated in the circulation immediately after the infusion (t_0) averaged 49, with a range of from 27 to 62.

Influence of various factors on the disappearance rate of labeled granulocytes. The procedure was varied with respect to concentration of DFP^{32}, temperature of incubation, volume of blood labeled, type of anticoagulant and container used, and the time of incubation. The influence of these procedural changes on the $T_{\frac{1}{2}}$ and on the percentage of infused cells which could be demonstrated in the circulation immediately after the infusion (t_0) was studied.

Varying the concentration of the DFP^{32} between 0.1 and 1.0 μg per ml had no influence on either the percentage of infused cells present in the circulation at t_0 or the $T_{\frac{1}{2}}$ (Table I). Incubating the blood at 1°, 20° and 37° C had no significant influence on these measurements (Table II). Likewise, labeling 100 or 250 ml of blood rather than 500 ml had no influence on the percentage of infused cells present at t_0 or on the $T_{\frac{1}{2}}$ (Table III). In single trials the use of heparin or sequestrene as an anticoagulant in place of ACD-A or the substitution of a siliconized glass container for the plastic container had no influence on these measurements.

Modifying the time of incubation from 15 minutes to 24 hours influenced significantly the percentage of infused cells present in the circulation at t_0 (Table IV). The proportion of the infused

TABLE II

Influence of incubation temperature *

Temperature	No. of subjects	Percentage of infused cells present at t_0 Mean	Range	$T_{\frac{1}{2}}$ Mean	Range
		%	%	*hrs*	*hrs*
37° C	4	57	38–68	7.6	6.0–8.7
20° C	8	49	27–62	6.9	5.5–8.0
1° C	3	44	31–54	5.5	4.4–6.1

* Five hundred ml of blood was incubated in a plastic bag with ACD-A containing 0.3 μg of DFP^{32} per ml for 60 minutes at the temperatures given above.

cells present in the circulation at t_0 decreased exponentially as the incubation period was prolonged (Figure 3). When blood was stored for more than 8 hours, less than 12 per cent of the infused cells was present in the circulation at t_0 and a disappearance curve could not be obtained.

No correlation was observed (correlation coefficient, -0.03) between the percentage of infused cells present in the circulation at t_0 and the $T_{\frac{1}{2}}$.

Transfusion of blood from normal subjects into normal recipients. Five hundred ml of blood was withdrawn from each of ten normal subjects. The blood was incubated for 60 minutes at room temperature with 0.3 μg of DFP^{32} per ml and then transfused into a normal recipient other than the donor. All subjects were cross-matched for the major blood group and Rh type. None of the subjects had ever received a blood transfusion prior to the experiment.

In seven of the subjects the granulocytes disappeared in an exponential fashion. The $T_{\frac{1}{2}}$ values in this group (5.4 to 9 hours) were within normal limits for autogenous infusions. In one subject the experimental values were widely scattered, presumably due to technical errors, and a line

TABLE III

Influence of the volume of blood labeled *

Volume of blood labeled	No. of subjects	Percentage of infused cells present at t_0 Mean	Range	$T_{\frac{1}{2}}$ Mean	Range
ml		%	%	*hrs*	*hrs*
100	1	49		7.3	
250	3	43	37–46	6.4	5.0–7.0
500	3	42	26–68	7.5	6.0–9.0

* The volumes of blood given above were incubated in a plastic bag with ACD-A for 60 minutes at room temperature with 0.2 μg of DFP^{32} per ml of blood.

TABLE IV

Influence of incubation time *

Time of incubation	No. of subjects	Percentage of infused cells present at t_0		$T_{\frac{1}{2}}$	
		Mean	Range	Mean	Range
hrs		%	%	*hrs*	*hrs*
0.25	3	47	26–81	5.3	5.0– 5.7
1	21†	41	14–72	7.0	5.2– 9.0
2	5	39	19–52	9.4	7.5–12.0
8‡	5	24	12–48	9.0	
16‡	5	12	5–21		
24‡	4	7	5– 9		

* Five hundred ml of blood was incubated at room temperature in a plastic bag with ACD-A containing 0.3 μg of DFP^{32} per ml of blood, with the exceptions noted.

† This group includes studies done with concentrations of DFP^{32} from 0.1 to 1.0 μg per ml.

‡ Incubated for 1 hour at room temperature and at 5° C for the remainder of the incubation period.

could not be drawn through the points. In two of the subjects the granulocytes disappeared from the circulation in an exponential fashion but at an accelerated rate ($T_{\frac{1}{2}} = 2.5$ and 1.2 hours).

A transfusion of blood from a normal subject was given to a patient with hemophilia. This patient had received approximately 100 previous transfusions of plasma or whole blood. The $T_{\frac{1}{2}}$ of the labeled cells in the circulation of the patient was 0.8 hour, in contrast to the normal mean of 6.6 hours. Twenty-two per cent of the transfused cells was present in the circulation of the patient at t_0.

DISCUSSION

This study was undertaken to determine the manner and rate of removal of normal granulocytes from the circulation of normal subjects. The technic described for labeling granulocytes *in vitro* has the advantage that only granulocytes circulating in the blood are labeled and, therefore, interpretation of the disappearance curves is more simple than interpretation of curves obtained following the intravenous administration of DFP^{32} (1).

Interpretation. With the method outlined, only about 50 per cent of the infused cells was present in the circulation at the completion of the infusion. Those cells which were not removed immediately disappeared from the circulation in an exponential fashion with a mean $T_{\frac{1}{2}}$ of 6.6 hours. Thus, from a kinetic point of view, the labeled granulocytes appeared to behave as two distinct cell populations. Several explanations for this phenomenon may be offered.

The immediate disappearance from the circulation of about 50 per cent of the labeled cells could be due to rapid elution of the label, rapid removal of leukocytes damaged by the labeling procedure, or to distribution of the infused granulocytes in a larger pool of cells than that calculated from the blood volume and the granulocyte count.

As the result of studies previously reported (1) the possibility of elution seems remote but cannot be entirely excluded. However, if the label eluted *in vivo*, it might be expected that the percentage of labeled cells present at t_0 would decrease as the concentration of DFP^{32} was increased. Such was not found to be the case (Table I).

The possibility of damage to the cells during the labeling procedure deserves serious consideration since granulocytes removed from the body are highly susceptible to damage and it has been shown that damaged granulocytes are removed by the lungs, possibly the first time they pass through the circulation (9–13). In the studies reported here, a greater proportion of the infused granulocytes was missing from the circulation when blood was stored for 8 to 24 hours and then infused than when blood was incubated for an hour or less (Table IV and Figure 3) and infused. It appears, however, that rapid removal of labeled cells which have been damaged as a function of the time of incubation is not the sole reason for the immediate

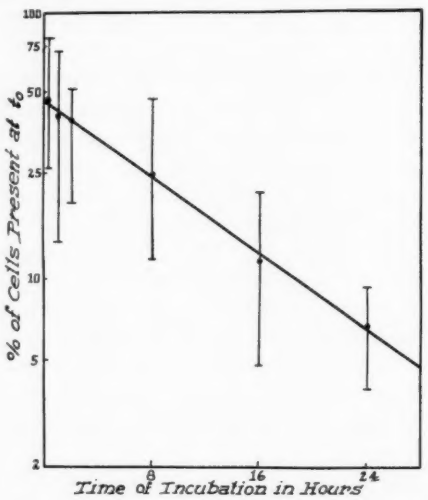

FIG. 3. THE EFFECT OF TIME OF INCUBATION ON THE PERCENTAGE OF INFUSED CELLS PRESENT IN THE CIRCULATION AT TIME ZERO (t_0).

disappearance of about half of the labeled granulocytes. Extrapolation of the exponential curve (Figure 3) to the ordinate, i.e., zero incubation time and therefore presumably zero damage, suggests that 54 per cent (100–46) of the infused cells was removed rapidly for a reason unrelated to the time of incubation.

The most plausible explanation for this rapid removal is that the labeled granulocytes are distributed in a pool of granulocytes larger than that calculated from the blood volume and the granulocyte count. Evidence in support of this concept will be presented in the next paper (14).

The exponential disappearance of labeled granulocytes from the circulation with a mean $T_{\frac{1}{2}}$ of 6.6 hours indicates that granulocytes leave the blood in a random fashion. That is, unlike erythrocytes, the disappearance of granulocytes from blood is unrelated to the time of entry of the cells into the circulation, and, by chance, a particular granulocyte may remain in the circulation only a few seconds or for an indefinitely long time. In this regard it is important that a correlation was not observed between the percentage of infused cells in the circulation at t_0 and the granulocyte disappearance rate ($T_{\frac{1}{2}}$). This suggests that the $T_{\frac{1}{2}}$ is representative of the disappearance rate of the entire labeled population of cells, rather than that of a selected population as, for example, the youngest cells. The validity of the $T_{\frac{1}{2}}$ is further substantiated by the observation (3) that granulocytes labeled *in vivo,* without any handling, also disappeared from the circulation in a random fashion and at the same rate as do cells labeled *in vitro* and then returned to the circulation. The mean $T_{\frac{1}{2}}$ of the cells labeled *in vivo* in 30 normal subjects was 6.4 hours; the mean $T_{\frac{1}{2}}$ for the cells labeled *in vitro* in the 45 normal subjects in this study was 6.6 hours. The difference between these mean values is not significant.

Application of the method. It is anticipated that this method will be useful in studying the granulocyte disappearance rate in leukemia and in other conditions. By the use of homologous transfusions in normal subjects, as illustrated by the ten preliminary studies reported herein, a reduction of the $T_{\frac{1}{2}}$ value may prove to be a highly sensitive method for detecting leukocyte incompatibilities and granulocyte blood groups. By a combination of autogenous infusion, transfusion of the patient's blood into a normal subject, and the transfusion of normal blood into the patient, it may become possible to differentiate granulocytopenias which are attributable to intracellular defects from those which may be produced by extracellular causes.

This method may also prove useful in the study of the preservation of granulocytes. Our studies clearly indicate that the longer granulocytes are stored, the more susceptible they become to immediate removal from the circulation. Ninety-one to 95 per cent of the granulocytes stored for 24 hours was removed immediately. Whether this is due to damage to the cells by the anticoagulant, by exposure to a foreign surface, by the depletion in the mixture of an essential nutrient, or by the accumulation in the stored blood of a product of cellular metabolism, is subject to experimental investigation.

SUMMARY

1. A method has been described for labeling granulocytes *in vitro* with radioactive diisopropylfluorophosphate (DFP^{32}). Briefly, the method is as follows: 500 ml of blood is withdrawn into a plastic bag containing acid citrate dextrose solution and incubated at room temperature for 1 hour with 0.3 μg of DFP^{32} per ml of blood. The blood containing the labeled granulocytes, erythrocytes and platelets is infused into the donor. At appropriate time intervals, 20-ml samples of blood are withdrawn. The leukocytes are separated free of erythrocytes and platelets and the leukocyte radioactivity is determined.

2. Fifty-four per cent of the labeled granulocytes disappeared from the circulation during the infusion. The remainder of the granulocytes was removed from the circulation in an exponential fashion; the mean half-time in the circulation was 6.6 ± 1.4 hours in 45 normal male subjects.

3. The proportion of labeled cells infused which was present in the circulation at the end of the infusion was not influenced by the temperature of incubation, the concentration of DFP^{32}, the volume of blood labeled, or the type of anticoagulant or container used, within the limits of the experiments performed. The proportion was influenced, however, by the time of incubation; the longer the blood was incubated, the smaller was the propor-

tion of infused cells in the circulation immediately on completion of the infusion.

4. The time for the disappearance of one-half of the labeled granulocytes was not influenced by the proportion of infused cells which was demonstrable in the circulation at the end of the infusion, or by the time or temperature of incubation, the concentration of DFP^{32}, the volume of blood labeled, or the type of anticoagulant or container used.

5. Ten normal male subjects were transfused with labeled blood from other normal subjects. In seven of the subjects, the granulocytes were removed at the normal rate. In two of the subjects, the granulocytes disappeared at an accelerated rate. In one subject, the experimental values were widely scattered and a line could not be drawn through the points.

6. The validity, limitations, interpretations and applications of this technic are discussed.

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MEASUREMENT OF SMALL QUANTITIES OF INSULIN-LIKE ACTIVITY USING RAT ADIPOSE TISSUE. I. A PROPOSED PROCEDURE *

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In the absence of sufficiently sensitive methods for the chemical determination of insulin, attempts to measure insulin in biological fluids have been almost entirely limited to bioassay procedures and, more recently, to immunological techniques. The bioassay procedures used have been based on the hypoglycemic effect of insulin in animals whose sensitivity was increased by endocrine manipulation (1–3) or on the effects of insulin on isolated tissue, in particular the mammary gland of lactating rats (4), and the diaphragm of rats (5–8) or mice (9). The largest body of information currently available concerns procedures based upon the effect of insulin on glucose uptake by rat diaphragm muscle (10).

The observation that isolated adipose tissue not only responds directly to insulin (11–13), but does so at very low insulin concentrations (14, 15), has indicated the possible usefulness of this tissue for the measurement of small quantities of insulin (15). It is the purpose of this and the following paper to present the information which has been accumulated in this laboratory over the past three years, during an attempt to elaborate and evaluate a suitable procedure, which might in time become applicable to tissue extracts or to biological fluids such as serum. Whenever applied to materials other than solutions of pure insulin, the term "insulin-like activity" (ILA) will be used in preference to the terms "insulin," "insulin-activity," or "effective insulin activity."

Being well aware of the many questions which are properly raised when a sensitive bioassay procedure is introduced and its application to complex solutions such as serum is suggested, the authors would like to separate the procedure itself from the question of its applicability to serum. This first paper will report the studies designed to select a procedure suitable for detecting *small amounts of pure insulin*. The second paper will present an analysis of the routine performance of the procedure when applied to solutions of pure insulin and to more complex solutions, using selected serum samples as specific examples.

METHODS

Selection of a suitable metabolic index

It is now established that, in the presence of insulin, adipose tissue incubated *in vitro* utilizes more glucose (11), incorporates more glucose into glycogen (16), oxidizes more glucose carbon to CO_2 (13, 16, 17), and incorporates more glucose carbon into fatty acids (13, 17) and glycerol (16). Furthermore, since insulin markedly increases overall CO_2 production (18) while affecting oxygen consumption only to a small degree (12, 18), the presence of insulin increases net gas production by this tissue. Any of these metabolic effects of insulin could be used as indices of ILA in a bioassay procedure. In this laboratory, some experience has been gained with the use of increased glucose uptake as an index for ILA (15), and Beigelman (19, 20), as well as Humbel (21), has published data concerned with the evaluation of a measurement based on glucose uptake. This measurement requires meticulous care in the handling of glucose estimations, since it frequently represents a small difference between two independent glucose measurements, each with its inevitable error. Ball, Martin and Cooper (18) have suggested that the use of net gas production by adipose tissue for the measurement of ILA would allow for the metabolic characterization of each tissue before the addition of insulin standards or unknown solutions, as

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FIG. 1. INCUBATION FLASK USED IN THESE STUDIES.

well as for its final characterization regarding its maximal responsiveness to insulin.

Among the other indices mentioned, CO_2 production from glucose carbon and incorporation of glucose carbon into fatty acids show the most favorable dose response relationships, i.e., the steepest dose response curves associated with similar precision (22). Of the two, CO_2 production from glucose labeled with C^{14} is more convenient as a measurement, since the procedures involved require less time and since less radioactivity is required because dilution of the labeled glucose in the large pool of adipose tissue lipids is avoided. Accordingly, the index which has been selected for the experiments reported here has been that of the formation of $C^{14}O_2$ from glucose labeled with C^{14}. Glucose-1-C^{14} has been used in preference to uniformly labeled glucose, since the oxidation of this carbon is particularly affected by insulin (17, 23) and since, as will be discussed below, the oxidation of this carbon is less affected by other metabolic agents such as epinephrine (24) or growth hormone (25).

Description of the procedure used

Animals and tissues. The animals used were male albino rats of the Wistar strain (Harvard Biological Laboratories and Albino Farms) fed *ad libitum* on Purina laboratory chow, weighing between 200 and 260 g. They were kept in individual cages for 2 to 5 days prior to use between 8 and 9 a.m. The tissue used was epididymal adipose tissue.

Incubation flasks. The flasks used for incubation were ordinary glass bottles with a total capacity of 35 ml. They were stoppered with sleeve-type rubber stoppers, the inner sleeves having a diameter of 25 mm. Small plastic cups (1 cm diameter and 1 cm high) were suspended from the inner sleeve with Chromel wires, to permit their easy removal and replacement (see Figure 1).

Incubation medium. The buffer solution used for incubation of the tissues was a modified Krebs bicarbonate buffer containing: Na, 146; K, 5; Ca, 2; Mg, 1; Cl, 114; and HCO_3, 40 mEq per L. The buffer was equilibrated with 95 per cent O_2, 5 per cent CO_2 bubbled for 10 minutes through the solution, and incubated in the presence of a 95 per cent O_2, 5 per cent CO_2 atmosphere. Under these conditions the pH was approximately 7.4. In addition, gelatin (USP, obtained from J. T. Baker Chemical's Corporation, Phillisburg, N. J.), 200 mg per 100 ml (approximate), was routinely added and was easily dissolved by bringing the buffer solution to 38° C. Glucose concentration (using glucose obtained from the National Bureau of Standards) was brought to 250 mg per 100 ml by the addition of dry glucose; 1.9 ml of buffer thus contained 4.75 mg of glucose.

Preparation of solution containing glucose-1-C^{14}. For each batch of tracer glucose, 200 μc of glucose-1-C^{14} (obtained from New England Nuclear Corporation and chromatographically pure) was dissolved in 50 ml distilled water. Nonradioactive glucose was added to bring the final glucose concentration of this solution to 1,250 mg per 100 ml; 0.1 ml of the solution thus contained 0.4 μc and 1.25 mg of glucose.

Preparation of insulin standards. A single batch of insulin has been used throughout these investigations (Crystalline insulin, five times recrystallized, treated with trypsin, batch no. 466368, containing 26.6 U per mg). This preparation was made available to us through the courtesy of Dr. W. R. Kirtley of the Lilly Research Laboratories. Once monthly, a concentrated insulin standard was prepared from this crystalline material by dissolving 10 mg in 10 ml of distilled water, a few drops of 1.0 N hydrochloric acid being added until the solution was clear. This concentrated standard thus contained 26.6 U per ml. It was stored at 4° C. Once weekly, 1 ml of the concentrated standard was brought to 26.6 ml with distilled water, a solution thus containing 1 U per ml. This working standard was again stored at 4° C.

Each day, 1 ml of the working standard was diluted to 100 ml by the addition of buffer containing glucose and gelatin as described above. Two ml of this solution was diluted to 10 ml with buffer containing glucose and gelatin. From this last solution serial dilutions were prepared using 10 ml of insulin-containing solution and 10 ml of buffer containing glucose and gelatin, resulting in solutions with 1,000, 500, 250, 125, 62, and 31 μU per ml. During these dilution procedures, buffer containing glucose and gelatin was always poured first into any glassware used for the preparation of insulin standards, in order to moisten and coat the walls of the containers with the gelatin-containing buffer, so as to prevent loss of insulin upon glassware.

Assay technique: Principle. Rat adipose tissue is incubated in bicarbonate buffer with glucose-1-C^{14} for 2 hours. At the end of the incubation, the total CO_2 present in the system is liberated by the addition of acid to the medium and is trapped in a small amount of NaOH. The sodium carbonate so obtained is transferred to a planchet, dried, and its radioactivity determined. The total radioactivity of the CO_2 is expected to be di-

rectly proportional to the amount of the first carbon of glucose oxidized to CO_2, and, as will be shown below, is further related to the amount of insulin present in the system.

Assay technique: Procedure. For each assay 18 flasks are used and into each was pipetted 1.9 ml of unknown solution [1] or of standard insulin solution in buffer containing glucose and gelatin as described above. Exactly 0.1 ml of the glucose-1-C^{14} solution was then added to each flask, which thus contained 2 ml of medium, 6 mg glucose (i.e., 300 mg per 100 ml) and 0.4 μc C^{14}. The flasks were weighed to the nearest milligram and kept stoppered to prevent evaporation.

After the flasks had been prepared and weighed, three rats were successively killed by a blow to the head, decapitation and exsanguination. The abdomen was widely opened and the testes were displaced by gentle pressure from the scrotum into the abdominal cavity. Each testis in turn was lifted with forceps and the adipose tissue hanging from the epididymis was cut free (single cut) just distal to the epididymal vessels, where the base of the epididymal fat is somewhat narrower. Without chilling, and with a minimum of handling, each pad was then taken up at its base and further cut into three equal segments.[2] As it was cut, each segment was dropped into its appropriate preweighed flask which had been kept stoppered. The segments were so distributed as to lead to the "balanced segment design" described below. The flasks were then rapidly reweighed to obtain the tissue weight. After gassing for 5 minutes with 95 per cent O_2, 5 per cent CO_2, using no. 21 hypodermic needles inserted through the rubber stoppers, the needles were withdrawn and the system was left airtight. The flasks were then incubated in a Dubnoff metabolic shaking incubator for 2 hours at 37° C, using 72 shaking cycles per minute (c/min).

After incubation, 0.1 ml of 1.8 N NaOH freshly diluted with CO_2-free water from saturated NaOH was injected into the suspended plastic cup and 0.2 ml of 10 N H_2SO_4 was added to the medium in each flask. Graduated tuberculin-type syringes fitted with no. 21 hypodermic needles were used for this procedure. The flasks were well mixed and agitated at 36 c/min for at least 60 minutes. The contents of each plastic cup were then transferred to a preweighed stainless steel planchet using two washes with 2 drops each of CO_2-free water. To insure even drying each planchet also contained a disc of lens paper weighing approximately 1 mg. For this transfer, the plastic cups are best handled with rubber-covered curved forceps.

The planchets were then dried on a hot plate, with care to avoid boiling and splattering of the planchet contents. After the contents appeared dry (approximately 0.5 hour), drying was continued for 1.5 hours at a somewhat higher setting of the plate. The planchets were cooled in a desiccator and reweighed. The radioactivity was estimated in a proportional flow counter. The counts ranged from several hundreds to several thousands per minute per planchet. The results are expressed as counts per minute per total CO_2 produced per milligram of adipose tissue, self-absorption corrections being applied according to Karnovsky and associates (26).

[1] For example, serum, glucose content having been brought to 250 mg per 100 ml by the addition of dry glucose.

[2] The segment closest to the base (i.e., closest to the epididymis) is termed the "proximal" segment, followed by the "medial" and the "distal" segments. The proximal segment did not include the portion which had been handled with forceps.

RESULTS

Evaluation of measurements and conditions

Adequacy of $C^{14}O_2$ collection. In the experiments shown in Table I, a measured amount of $NaHC^{14}O_3$ was added to 2 ml of either buffer or serum, $C^{14}O_2$ collection then being carried out as described above. Intervals of 30, 60 and 180 minutes were allowed to elapse between the addition of acid to the buffer or serum, and the transfer of the alkali from cups to planchets. Furthermore, two normalities were used for the 0.1 ml NaOH contained in the cup: 1.8 and 0.9 normal. Theoretically, the normality required to neutralize the carbonic acid formed would be between 0.6 and 0.8 for the 0.1 ml volume used. The results obtained indicate that the recovery of $C^{14}O_2$ was maximal from 30 minutes on, although this was true only when the NaOH added to the cup was 1.8 N, and that comparable recoveries were obtained from buffer and from serum. It is necessary to stress the need for heating the CO_2-containing alkali on the planchets, a need probably explainable on the basis of the hygroscopy of the excess NaOH. Furthermore, complete drying on the hot plate

TABLE I

Recovery as $C^{14}O_2$ of $NaHC^{14}O_3$ added to buffer or serum

Medium	Collection period	NaOH (normality)	Weight on planchets	Total cpm*
	min		*mg*	
Buffer	30	1.8	11.8	859
Serum	30	1.8	11.4	841
Buffer	60	1.8	11.1	813
Serum	60	1.8	11.5	870
Buffer	180	1.8	11.5	855
Serum	180	1.8	11.6	810
Buffer	180	0.9	5.9	606
Serum	180	0.9	5.8	614

* Each figure represents the mean of two experiments.

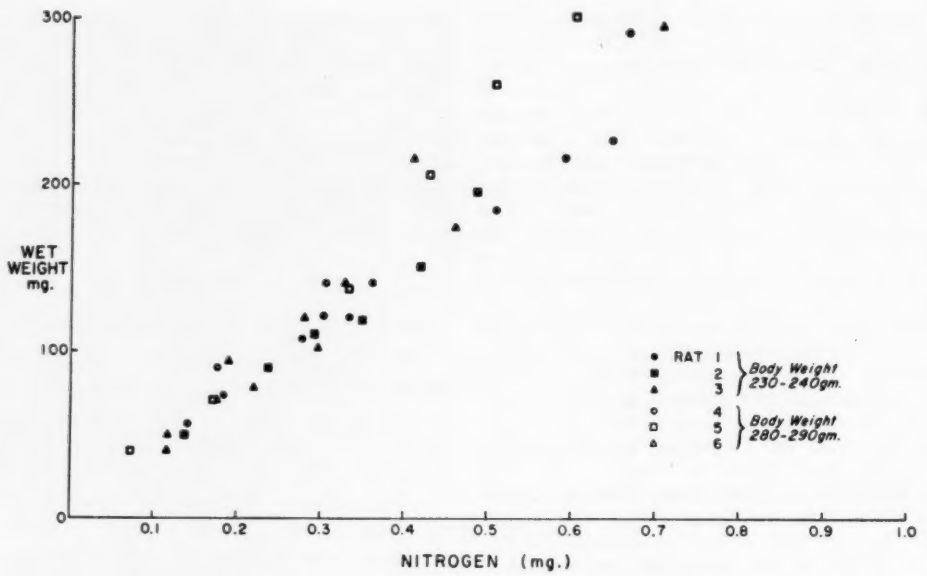

FIG. 2. RELATION OF WET WEIGHT OF ADIPOSE TISSUE SEGMENTS TO THEIR NITROGEN CONTENT.

insures even weights and decreases the importance of accepting the validity of the correction factor used to correct for self-absorption in calculating the radioactivity contained in the sample.

Reference measurement. Reference measurements which might be considered in this tissue are primarily wet weight, dry fat-free weight, or nitrogen. Dry fat-free weight of fragments of tissue of the size used averaged between 1 and 2 mg, a range inconvenient for accurate measurement. Wet weight and total nitrogen content have been compared in a large series of experiments of which six are shown in Figure 2. Although the ratio of nitrogen to wet weight varies from animal to animal, it is apparent from the figure that it remains relatively constant within tissues obtained *from a single animal.* It would seem, therefore, that wet weight of adipose tissue permits reasonable quantitation of the active tissue components contained in each fragment.

Duration of the incubation. Winegrad and Renold (13) have shown that rat adipose tissue incubated either with or without insulin for periods varying from 15 minutes to 6 hours metabolizes glucose to CO_2 at approximately the same rate for the first 3 hours with some decrease after that time. Using net gas formation in the presence of glucose, Ball and co-workers (18) reported constant activity of the tissue up to 6 hours. Piazza, Goodner and Freinkel (27) have recently reported that adipose tissue releases trichloroacetic acid-soluble I^{131} when incubated with insulin labeled with I^{131}, although to a lesser extent than is true for diaphragm. Whereas the activity studies just mentioned do not suggest a significant destruction of insulin-like activity for periods of incubation up to 3 hours, it is evident that this possibility must be considered in each instance.

Ionic environment. Zahnd, Dagenais and Renold [3] have studied in detail the ionic requirements both for baseline activity of rat adipose tissue and for the insulin response, and found that the baseline activity of the tissue is quite markedly affected by variations in both the cationic and the anionic environment. Lack of potassium, magnesium and calcium resulted in increased baseline activity, while the level of activity reached in the presence of insulin was somewhat depressed. As a result, the insulin effect was considerably smaller. Baseline activity, insulin-stimulated activity, and insulin effect all were much smaller when phosphate was substituted for bicarbonate. No variation in the ionic environment has yet been found which leads to an increased insulin effect, and use

[3] Unpublished observations.

of the Krebs bicarbonate buffer which we have adopted appears, for the present, optimal. Hagen, Ball and Cooper (28), using net gas production as an index of tissue activity, came to similar conclusions.

Concentration of glucose. Jeanrenaud and Renold (22) have studied in some detail the interrelations of the effects of glucose concentration and insulin concentration upon rat adipose tissue. For any given dose of insulin a greater response was obtained when the concentration of glucose was greater, the response being roughly proportional to the logarithm of the glucose concentration. Accordingly, the relatively high glucose concentration of 300 mg per 100 ml has been selected for the procedure reported here. The primary point is to keep glucose concentration constant in any individual assay. Whenever a sample with a glucose concentration higher than 300 mg per 100 ml was assayed, the glucose concentration for both the standards and all unknowns was similarly increased.

The addition of gelatin. The routine addition of gelatin to all solutions used in these studies has been prompted by the need to avoid loss of insulin on glassware, a loss which has been well documented at extremely low concentrations (29, 30), and which we have confirmed.

Weight of animals. Ball and associates (18) have demonstrated a rather striking relationship between the weight of animals and the net gas formation by their adipose tissue. As marked a relationship has not been observed when $C^{14}O_2$ production from glucose-1-C^{14} was the metabolic index. It seemed, however, that tissues were more frequently below average activity when animal weights of 260 g were exceeded, and greater than usual fluctuations in activity were observed in animals weighing less than 200 g. Furthermore, when animals weighing less than 220 g were used, it was frequently impossible to obtain sufficient tissue to remain within the optimal weight range for each fragment. Accordingly, for the animals of the particular strain used, a weight range of 220 to 260 g is suggested. It is conceivable that limitation to a smaller weight range would be advantageous, although this causes practical difficulties.

Weight of tissue fragments. When the activity was expressed in counts per minute per milligram of tissue, analysis of well over 600 individual observations indicated that the variation of activity of segments weighing between 70 and 170 mg was similar and did not show a trend relating it to segment weight. Below 70 mg, activity expressed per milligram of tissue tended to be higher, and above 170 mg, it tended to be lower than between these two values. Accordingly, it is suggested that tissue fragments be kept within this general weight range.

Differences between segments and adequacy of routine procedures such as pipetting. The basic experimental unit used in all assays is a segment (approximately one-third) of each epididymal fat pad. To determine whether systematic differences exist between the responses observed in the different parts of each fat pad, i.e., in the distal, medial and proximal segments (see Footnote 2), an experiment was performed using 12 rats, three on each of four successive days. On the first and third days, all the segments from one fat pad of each rat were incubated with buffer only and those from the other pad with 62 μU per ml of standard insulin. On the intervening days, the treatments were buffer and 1000 μU per ml of insulin. The differences between the geometric means of the counts per minute per milligram were evaluated by an analysis of variance (31) of the log counts per minute. On three days out of the four, differences between individual rats were significantly ($p < 0.001$) greater than the residual error term.

TABLE II

Response of epididymal fat pads of rats by segment *

Experiment day	Insulin dose	Geometric mean		
		Distal segment	Medial segment	Proximal segment
	μU	*cpm/mg*	*cpm/mg*	*cpm/mg*
1	0	5.6	6.8	5.8
3	0	6.9	6.0	5.4
Mean		6.2	6.4	5.6
1	62	15.6	12.8	8.9
3	62	16.6	13.4	12.6
Mean		16.1	13.1	10.6
2	0	4.5	5.9	4.4
4	0	4.3	5.1	4.7
Mean		4.4	5.5	4.6
2	1,000	16.7	18.5	16.0
4	1,000	26.8	26.6	24.3
Mean		21.2	22.2	19.7

* Each value is derived from the corresponding segments from three rats.

TABLE III

Experiment to compare adipose tissue segments, preparation of insulin solutions (A and B), and order of pipetting (1, 2, 3) *

Insulin (62 μU)	Distal segment A	Distal segment B	Medial segment A	Medial segment B	Proximal segment A	Proximal segment B	Geometric mean
Experiment day†							
Pipetting	1		2		3		
1	12.4	10.3	10.7	10.2	9.6	10.6	10.6
2	9.5	7.6	9.0	9.4	5.0	6.4	7.6
3	19.5	15.6	18.4	12.8	10.2	22.3	16.0
4	30.1	23.4	33.0	23.8	21.7	21.1	25.1
Pipetting	2		3		1		
1	33.8	39.2	30.0	25.9	19.8	18.0	27.8
2	44.0	45.9	32.5	39.6	35.9	29.9	37.5
3	11.1	15.7	12.0	21.6	11.3	14.6	13.9
4	32.8	43.1	32.1	39.9	31.4	31.1	34.8
Pipetting	3		1		2		
1	53.9	40.3	32.3	21.3	26.8	23.8	31.3
2	38.8	26.0	37.5	31.7	21.7	25.0	29.5
3	9.5	14.5	9.6	13.1	8.6	11.5	12.7
4	20.3	17.3	22.1	19.1	18.6	15.8	18.8
Geometric mean	22.4	21.4	20.6	20.2	15.8	17.5	
Mean log cpm/mg‡	1.35	1.33	1.31	1.30	1.20	1.24	

Geometric means by insulin preparations and sides of rats:

	A	B	Both
Left side	21.3	19.8	20.4
Right side	17.6	19.4	18.6
Both sides	19.4	19.6	

* Each line represents the responses, in cpm per mg, from one rat.

† On Days 1 and 2, insulin preparation A was used for segments from the left side of the rat and preparation B for segments from the right side. This assignment was reversed on Days 3 and 4.

‡ Standard error of each mean was 0.034, as calculated with the treatment X day interaction as error term.

Segment differences (Table II) were less pronounced, but the combined responses from the proximal segments were significantly lower ($p < 0.01$) than from the other segments. The differences between the medial and distal segments were inconsistent and were significant ($p < 0.05$) on one day only.

Again with the use of three rats on each of four successive days, a factorial experiment (31) was performed to study the effects of: *a*) segment differences as indicated by the results just reported; *b*) variability in the preparation of the standard insulin solution; *c*) repeated use of the same pipet for any one solution; and *d*) differences between right and left fat pads of the same rats. Two preparations of insulin (A and B) were made by independent serial dilutions starting from the same standard solution containing 26 U per ml. Each piece of tissue was incubated with 62 μU of insulin taken from either preparation A or preparation B. The order of pipettings was designated as 1, 2 or 3, the order being balanced over all segments on any one day.

As shown in Table III, the proximal segments were again found to yield significantly ($p < 0.01$) lower values than the other segments; differences between rats were also highly significant ($p < 0.001$). On the other hand, no significant effect of insulin preparation, sides, or pipettings was observed. These results led to the adoption of the "balanced segment design" discussed in the following paper.

DISCUSSION

Sensitivity

That insulin concentrations as low as 10 μU per ml, in the presence of gelatin-containing buffer, significantly enhance the production of $C^{14}O_2$ from glucose-1-C^{14} by the tissue preparation used is shown in Table IV.

Relationship between dose and response and choice of a statistical response metameter

Dose-response relationships observed in six animals are shown in Figure 3. Although the data are obtained as total counts per minute per milligram wet weight (Figure 3A), it is desirable that they be transferred into some other units to meet the following requirements (*32*): *a*) similar absolute biological and experimental variation at different response levels as measured by the "variance" or square of the standard deviation (this requirement is referred to in statistical terms as "homoscedasticity"); *b*) an additive, linear response to increasing doses of insulin within a suitable range.

Analyses were made of the square roots of the counts per minute per milligram and of the logarithms of the counts per minute per milligram to see whether or not these "metameters" gave satisfactory results. As illustrated by the examples in Figure 3, the requirement of linearity was met reasonably well with each of these metameters in the analysis of a number of dose-response curves. While the square root responses (Figure 3B) exhibited greater scatter at high concentrations of insulin than at low concentrations, this difficulty was avoided when the log counts per minute (Figure 3C) were used. To provide a further

TABLE IV

*Effect of 10 μU insulin per ml gelatin-containing medium upon the oxidation of glucose-1-C^{14} to $C^{14}O_2$ **

Animal no.	Radioactivity of CO_2 produced: A (without insulin)	B (with insulin)	Ratio B/A
	cpm/mg	*cpm/mg*	
1	0.19	0.32	1.68
	0.18	0.30	1.67
	0.22	0.24	1.09
2	14.1	16.0	1.14
	13.8	18.0	1.30
	15.5	20.0	1.29
3	9.2	12.9	1.40
	10.4	12.1	1.16
	11.1	15.5	1.40
4	12.8	14.9	1.16
	12.7	17.8	1.40
5	7.62	10.1	1.33
	8.15	10.3	1.26
6	19.1	22.4	1.17
7	2.80	3.66	1.31
8	1.40	2.31	1.65
9	3.59	8.81	2.45
10	14.8	18.8	1.27
11	25.5	28.2	1.11
12	5.93	8.01	1.35
Mean			1.38
SE			0.08
Significance			p† < 0.01

* Comparison was between matched segments from right and left epididymal pads.

† Probability that observed ratio could be a random sample from a population with a true ratio of 1.00.

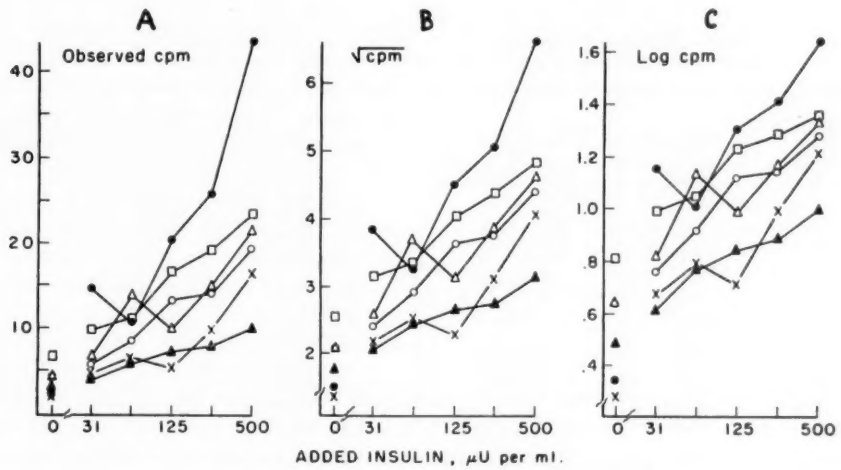

FIG. 3. DOSE–RESPONSE CURVES OBTAINED IN SIX ANIMALS PLOTTED WITH THREE DIFFERENT RESPONSE METAMETERS. The responses are expressed per milligram wet weight of tissue. The doses of insulin are plotted on a logarithmic scale.

TABLE V

*Effect of purified human albumin * and γ-globulin * on the oxidation of glucose-1-C^{14} by rat adipose tissue*

	No. of values	Radioactivity of CO_2 produced
		cpm/mg
Insulin (31 μU/ml)	6†	7.58
Human albumin (40 mg/ml)	12†	5.92
Human γ-globulin (10 mg/ml)	12	4.99
Insulin (1,000 μU/ml)	6	36.08

* Obtained from the Protein Foundation, Jamaica Plain, Mass., through the courtesy of Dr. Robert B. Pennell and Dr. H. N. Antoniades. Dialyzed against bicarbonate buffer prior to use.

† In each of six rats, single high and low insulin standards were compared with duplicate samples of albumin and globulin.

comparison, the index of precision ($\lambda = s/b$) for other assays was calculated with both metameters. The logarithmic transformation resulted in lower values of λ for 13 of 18 assays. The consistency from assay to assay was compared by calculating the standard deviations of the observed values of s (standard deviation) and of b (slope of the line). The logarithmic analyses resulted in somewhat smaller coefficients of variation (37 against 65 per cent for s, 34 against 37 per cent for b). The logarithmic transformation was therefore chosen as the routine method for subsequent analyses.

Specificity

That the effects of insulin upon adipose tissue are not completely specific is an *a priori* assumption of these studies. Just as many agents affect blood glucose levels in the intact animal, or glucose uptake by the isolated hemidiaphragm, many agents are likely to affect glucose metabolism by adipose tissue. The information to be presented in this section is intended merely to outline the general area of the specificity problem.

Since insulin is a protein, it seemed of interest to document whether proteins in general accelerate glucose oxidation by isolated adipose tissue. Table V demonstrates that purified human albumin or γ-globulin exerted less insulin-like action upon this tissue than the low standard (31 μU per ml), even at concentrations approximating those of serum or plasma. It is important to point out here that commercial preparations of these and other proteins frequently contain relatively large amounts of the agents used during their isolation, and that adequate dialysis is required prior to their use in the presence of isolated tissues.

The effects of glutathione are shown in Table VI. In the disulfide form, this peptide did not influence the oxidation of the first glucose carbon by adipose tissue, nor did it affect the action of added insulin. In the sulfhydryl form, however, glutathione alone, at the relatively high concentra-

TABLE VI

*Effect of oxidized and reduced glutathione (20 mM) on the formation of $C^{14}O_2$ from glucose-1-C^{14} by rat adipose tissue, with and without added insulin (250 μU per ml) **

Animal no.	No addition	Glutathione (oxidized)	Glutathione (reduced)	Insulin	Insulin and glutathione (oxidized)	Insulin and glutathione (reduced)
1	3.61†	5.39		39.55	23.08	
2	5.07	4.71		28.26	49.80	
3	4.57	5.82		31.07	28.66	
4	5.62	8.05		51.65	36.55	
5	9.16	6.23		27.44	28.61	
Mean	5.61	6.04		35.59	33.34	
6	5.21		16.57	60.20		15.48
7	6.00		12.50	42.93		15.68
8	5.05		10.86	22.86		10.31
9	3.59		8.60	14.70		8.10
10	4.10		10.70	13.20		10.00
11	5.50		8.80	12.10		9.30
Mean	4.91		11.34	27.68		11.48

* Glutathione, with or without insulin, was added to the buffer 2 hours prior to the addition of adipose tissue, and kept at room temperature during that time. The samples not containing glutathione were treated identically.

† Radioactivity of CO_2 produced (cpm/mg).

TABLE VII

Effect of serum (0.1 ml per ml) from normal guinea pigs and from guinea pigs immunized with beef insulin upon the formation of $C^{14}O_2$ from glucose-1-C^{14} by rat adipose tissue, with and without added insulin (250 μU per ml)

Animal no.	No addition	Normal serum	Anti-insulin serum	Insulin	Insulin and normal serum	Insulin and antiserum
1	2.85*		23.02	64.84		15.39
2	3.39		12.49	48.96		14.24
3	3.98		10.49	26.91		6.52
4			6.76	18.10		6.25
5			12.48	36.06		12.51
6			10.12	27.65		8.49
Mean	3.41		12.56	37.09		10.56
7	4.02	31.06		51.88	44.29	
8	3.94	10.84		28.22	32.31	
9	3.99	15.16		21.81	33.31	
Mean	3.98	19.02		30.64	36.64	

* Radioactivity of CO_2 produced (cpm/mg).

tion used, had an "insulin-like" effect, while completely neutralizing the effect of added insulin. The latter observation is in keeping with the known insulin-inactivating effect of reduced glutathione and of cysteine. The stimulating effect of glutathione alone is as yet unexplained. The suggestion that certain amino acids and peptides alter lipogenesis from acetate by adipose tissue (33), a metabolic event likely to be associated with altered rates of oxidation of the first glucose carbon, is but one of several possible explanations, all hypothetical.

The effects of reduced glutathione upon this tissue were studied in part because of its possible usefulness in the characterization of insulin-like substances in complex solutions. The experiments shown in Table VII were similarly motivated. Anti-insulin serum [4] was obtained by immunizing guinea pigs against beef insulin according to the procedure described by Moloney and Coval (34). It is evident from the data that 0.1 ml of the antiserum per ml of medium exerted a small but significant "insulin-like" effect upon adipose tissue (presumably endogenous insulin-like activity of guinea pig serum) while completely neutralizing the activity of 250 μU added beef insulin.

It is of importance to consider whether hormones other than insulin exert "insulin-like" or "anti-insulin-like" effects upon the adipose tissue preparation used. Present information available to us may be summarized as follows. *Epinephrine* markedly affects adipose tissue metabolism in general and, in particular, stimulates glucose uptake, glucose oxidation, and glyceride-glycerol synthesis by this tissue. However, glucose oxidation by the phosphogluconate-oxidative pathway, as well as lipogenesis, remains relatively unaffected (24, 35). As a result, the oxidation of the first carbon of glucose is little affected by epinephrine, at least at physiological concentrations of the hormone, as shown in Table VIII. This contrasts with the well documented antagonistic effect of epinephrine in the rat hemidiaphragm assay (36). *Growth hormone* (25, 37) and *adrenocorticotropin* (37–39) have been reported to influence glucose metab-

TABLE VIII

Effect of physiological concentrations of epinephrine upon the formation of $C^{14}O_2$ from glucose-1-C^{14} by rat adipose tissue, with and without added insulin (250 μU per ml).

Treatment	Animal no. 1	2	3	Mean
No addition	2.93*	4.02	2.94	3.30
Epinephrine				
1.8 μg/L	4.98	4.63	2.35	3.99
18 μg/L	3.96	4.42	2.97	3.78
Insulin	12.49	23.94	7.63	14.69
Insulin + epinephrine				
1.8 μg/L	11.92	21.81	7.09	13.61
18 μg/L	14.97	19.04	9.85	14.62

* Radioactivity of CO_2 produced (cpm/mg).

[4] The anti-insulin serum used in these experiments was generously made available to us by Drs. Werner Creutzfeldt and Paul Lacy, St. Louis, Mo.

olism by rat adipose tissue *in vitro* in a manner similar to that of epinephrine. Again, however, an insulin-like effect of these hormones appears unlikely under physiological conditions, in view of the concentrations required. Human growth hormone, in the concentration range of 0.1 to 5.0 μg per ml, and ACTH in the concentration range of 0.1 to 100 mU per 100 cc do not effect the oxidation of the first carbon of glucose by this tissue, either by itself or in combination with insulin.[5] The insulin-like effects of *glucagon* upon adipose tissue are markedly diminished by pretreatment with cysteine, suggesting that they are at least in part attributable to contamination with insulin (40). *Prolactin* has been shown to affect adipose tissue metabolism in a manner similar to that of insulin (*27*, 41), although at concentrations which are several orders of magnitude greater than those required for insulin. *Glucocorticoid* effects upon glucose metabolism by adipose tissue have been sought, but not found by us (*42*) although contrary observations have been reported very recently (43–45).

The authors suggest that the information so far available has failed to reveal hormonal activity other than that of insulin which is likely to interfere significantly with the determination of ILA in samples containing physiological amounts of these activities, while recognizing the limitation of this argument. On the other hand, the studies which have been mentioned, as well as others, clearly indicate that adipose tissue is a tissue whose metabolic balance is delicately controlled by a number of agents, including hormones. Another example concerns the reported ILA of nicotinic acid at concentrations which may occur in blood during therapy with this agent (40). It seems evident that whenever "insulin-like" activity, as defined in this report, is found in complex biological samples, a great deal of additional evidence will be required before identity of this activity with insulin can be assumed.

SUMMARY

A bioassay procedure suitable for measuring small quantities of insulin or "insulin-like activity" has been presented. The procedure is based upon the responsiveness to insulin of isolated epididymal rat adipose tissue. Several indices of insulin-like activity may be used, including glucose uptake, net gas formation, and lipogenesis from glucose labeled with C^{14}. The index selected has been that of production of $C^{14}O_2$ from glucose-1-C^{14}.

The method is capable of detecting 10 μU of insulin per ml. The relationship between the logarithm of the insulin concentration and the logarithm of the response appears to be linear and is analyzed further in the subsequent paper. An advantage of the procedure derives from the ability to compare several standard solutions and unknown samples within tissue from any one animal. It is sufficiently convenient to allow for the routine comparison of two standards and four unknown samples in each of three rats on any given day.

The effect of beef insulin upon this tissue is inhibited by pretreatment with reduced glutathione or by addition of antiserum obtained from guinea pigs. Although the tissue is responsive to many metabolic agents, including those of endocrine origin, information available so far has failed to reveal hormonal activities other than those of insulin which are likely to interfere significantly with the determination of insulin-like activity in samples containing physiological concentrations of these activities. It is essential, however, to consider any measured activity other than that derived from added crystalline insulin as "insulin-like" until sufficient evidence can be produced to establish its identity with insulin.

[5] Steinke, J., Zahnd, G., and Renold, A. E. Unpublished observations reported to 42nd annual meeting of the Endocrine Society, Miami, June, 1960, page 58 of program.

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MEASUREMENT OF SMALL QUANTITIES OF INSULIN-LIKE ACTIVITY USING RAT ADIPOSE TISSUE. II. EVALUATION OF PERFORMANCE *

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In the preceding paper (1), a biological assay method was proposed which utilized the increased production of CO_2 from glucose in response to minute quantities of insulin by epididymal adipose tissue of rats. Since the quality of an assay method is best revealed by its performance over a period of time, the present report will present the results obtained during 25 months when this assay procedure was applied to insulin solutions and was tested on more complex material such as human blood serum. The quantitative evaluation of the data was carried out to a large extent independently from the actual performance of the assays, although consultations between the statistical and the biological teams occurred at irregular intervals of a few months.

MATERIALS AND METHODS

Blood sera. Blood samples were collected by venipuncture and allowed to clot at room temperature for 1 to 2 hours. After centrifugation at room temperature, the sera were separated and immediately frozen after removing a small sample for glucose determination. The samples were then kept frozen until the evening preceding the assay when they were transferred to a cold room kept at 4° C. When repeated determinations were to be carried out on the same serum sample, aliquots were frozen separately in order to avoid repeated thawing and freezing.

Assay design. The technical details of the assay procedure have been described in the preceding paper (1). In a routine assay of unknowns, the standard insulin solution was always given at least at two concentrations: ≥ 31 μU and $\leq 1{,}000$ μU per ml. Four "unknowns" were usually assayed at single levels. The six "treatments" were each given to a tissue segment from each of three rats. Subsequent to the demonstration (1) of systematic differences between proximal, medial and distal segments,[1] a "balanced segment" design (illustrated in Table I) was adopted to eliminate these differences as well as differences between rats, from comparisons between treatments. The balanced segment design was also used in studies of the log dose-response curve (Table I).

Statistical methods. Except for some early analyses, the data were recorded as the logarithms (logs) of the counts per minute per milligram wet weight of adipose tissue (1). The calculations for an assay are illustrated in Figure 1. The log counts per minute per milligram were first subjected to an analysis of variance to derive the residual standard deviation (s) after removing the effect of differences between rats, between segments, and between treatments. The standard slope (b), calculated for each assay separately, was used to estimate, by accepted methods (2, 3), the logarithm (M′) of the potency of an "unknown" substance relative to the mean log dose ($\bar{x}_s$) of insulin standard. The logarithm (M) of the estimated insulin-like activity (ILA) in microunits of insulin per milliliter was calculated from M′ as shown in

TABLE I

Examples of design for segment balance over treatments within assays

Routine assay	Standard curve	Rat 1	Rat 2	Rat 3
	μU insulin			
Unknown 1	31	D*	P*	M*
Insulin, low dose	62	M	D	P
Unknown 2	125	P	M	D
Unknown 3	250	D	P	M
Insulin, high dose	500	M	D	P
Unknown 4	1,000	P	M	D

* D, P and M designate distal, proximal and medial adipose tissue segments, respectively.

[1] The segment closest to the base (i.e., closest to the epididymis) is termed the "proximal" segment, followed by the "medial" and the "distal" segments.

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‡ Recipient of a research fellowship from the American Diabetes Association.

§ Recipient of a research fellowship from the United States Public Health Service.

Response Metameter: y = log cpm Assay Date 8-28-59

RESPONSE:

RAT	Standard: Lo: 31	Hi: 1000	U1	Unknowns: U2	U3	U4	Sum(R)	Segment	Sum(S)
1	M 1.25	M 1.88	D 1.16	P 1.60	D 1.89	P 1.76	9.54	D	8.75
2	D 0.70	D 1.48	P 0.96	M 1.36	P 1.34	M 1.46	7.30	M	9.09
3	P 1.07	P 1.78	M 1.34	D 1.56	M 1.80	D 1.96	9.51	P	8.51
Sum(T)	3.02	5.14	3.46	4.52	5.03	5.18	26.35 = (Σy)		
$\bar{y}$	1.007	1.713	1.153	1.507	1.677	1.727			

BASIC CALCULATIONS:

$\Sigma(y^2)$: 40.6751 $\Sigma(R^2)/6$: 39.1236

$(\Sigma y)^2/N$: 38.5735 $\Sigma(S^2)/2r$: 38.6018

$\Sigma(T^2)/r$: 40.0251

$b = \frac{\bar{y}_{Hi} - \bar{y}_{Lo}}{\bar{x}_{Hi} - \bar{x}_{Lo}} = \frac{0.706}{1.509} = 0.468$

$\lambda = s/b$: .203

ANALYSIS OF VARIANCE:

Source	d.f	SS	MS	F	P
Total	17	2.1016			
Rats	2	0.5501	.2750	30.56	<.001
Segments	2	0.0283	.0142	1.58	>.05
Treatments	5	1.4516			
Regr.(std)	(1)	0.7491	.7491 = SSR	83.23	<.001
Residual	8	0.0716	.0090 = s^2		
			.095 = s		

POTENCY ESTIMATE:

		U1	U2	U3	U4
(1)	$\bar{y}_u - \bar{y}_s$	-.207	+.147	+.317	+.367
(2)	(1)/b = M'	-.442	+.314	+.677	+.784
(3)	(2) + $\bar{x}_s$ = M	1.804	2.560	2.923	3.030
(4)	$\log^{-1}$(3) = R	64	363	838	1071

$\bar{y}_s$: 1.360

$\bar{x}_s$: 2.246

"EXPANDED" STANDARD ERROR OF M:

$t^2_{.05}$: 2.306 $t^2_{.01}$: 5.318

$g = s^2 t^2/SSR$: .064

$1/(1-g)$: 1.068

$(N_s+N_u)/N_s N_u$: .5

(5)	$[(1)^2/SSR][1/(1-g)]$	.061	.031	.143	.192
(6)	$\frac{1}{1-g}\left[(5) + \frac{N_s+N_u}{N_s N_u}\right]$	.599	.567	.687	.739
(7)	$\sqrt{(6)}$	.774	.753	.829	.860
(8)	λ(7) = "Expanded" SE_M	.157	.153	.168	.175

CONFIDENCE LIMITS:

(9)	t(8) = 1/2 L	.362	.353	.387	.404
(10)	[1/(1-g)](2)	-.472	+.335	+.723	+.837
(11)	(10) + $\bar{x}_s$	1.774	2.581	2.969	3.083
(12)	(11) - (9)	1.412	2.228	2.582	2.679
(13)	(11) + (9)	2.136	2.934	3.356	3.487
(14)	$\log^{-1}$(12) = R_{Lo}	26	169	382	478
(15)	$\log^{-1}$(13) = R_{Hi}	137	858	2270	3069

FIG. 1. THE STATISTICAL ANALYSIS OF A ROUTINE ASSAY.* This analysis form is designed for routine assays in which six "treatments," two doses of insulin standard and four "unknowns," each at one level, are given to the adipose tissue segments of each of three rats. Modifications in the form and the analysis must be made if there are one or more missing observations (8) or if there is a different arrangement of treatments, i.e., more than two dose levels of insulin or more than one dose level of an unknown.

NOTATION: y = an individual response (log cpm/mg). x = log dose of insulin. $\bar{y}$, $\bar{x}$ = mean of y and x, respectively. D, M, P = distal, medial and proximal segment of fat pad, respectively. R = in the analysis of variance, sum of y for one rat. R, R_{Lo}, R_{Hi} in computation of potency and confidence limits, μU ILA. r = number of rats or of responses to one "treatment." T = sum of y for one "treatment." S = sum of y for a segment (D, M or P). N = 6r, total number of responses. N_s = 2r, number of responses to insulin standard. N_u = r, number of responses to a specific unknown. Σ = "the sum of." $t_{0.05}$, $t_{0.01}$ = value of t at the significance level selected (p = 0.05, p = 0.01) for the available degrees of freedom (d.f.) in the residual mean square (s^2). SS = sum of squares. MS = mean square (SS divided by d.f.). F = variance ratio (5), i.e., specific MS divided by residual MS.

* FORMULAS FOR THE ANALYSIS OF VARIANCE

Source	d.f.	SS	Example
Total	N-1	$\Sigma(y^2) - (\Sigma y)^2/N$	$(1.25^2 + \cdots + 1.96^2) - 26.35^2/18$
Rats	r-1	$\Sigma(R^2)/6 - (\Sigma y)^2/N$	$(9.54^2 + \cdots + 9.51^2)/6 - 26.35^2/18$
Segments	3-1	$\Sigma(S^2)/2r - (\Sigma y)^2/N$	$(8.75^2 + \cdots + 8.51^2)/6 - 26.35^2/18$
Treatments	6-1	$\Sigma(T^2)/r - (\Sigma y)^2/N$	$(3.02^2 + \cdots + 5.18^2)/3 - 26.35^2/18$
Regr. (std)	(1)	$(T_{Hi} - T_{Lo})^2/2r$	$(5.14 - 3.02)^2/6$
Residual	N-r-7	Total SS − (Rat + Segment + Treatment SS)	2.1016 − (0.5501 + 0.0283 + 1.4516)

Test for regression: F = regression MS/residual MS = 83.23, $p < 0.001$. If the regression F ratio is not significant, potency estimates cannot be made.

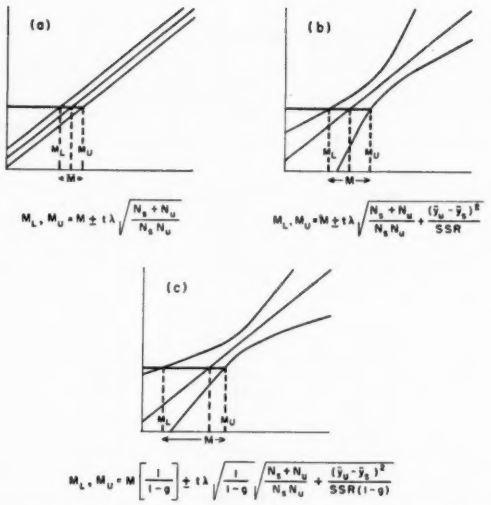

FIG. 2. SCHEMATIC ILLUSTRATION OF DIFFERENT METHODS USED IN CALCULATING CONFIDENCE LIMITS FOR M. The three diagrams show the same dose-response regression line, surrounded by confidence limits (for the line) that correspond to the formula illustrated. a. APPROXIMATE LIMITS WHICH NEGLECT ERROR IN SLOPE. b. WIDER APPROXIMATE LIMITS, BASED ON CALCULATIONS THAT IGNORE THE STATISTIC c. The confidence limits around the regression line are equally spaced in a horizontal direction from each point on the line, and M_L and M_U are symmetrically placed about M. c. ACCURATE LIMITS COMPUTED FROM THE RIGOROUS FORMULA (4), WHICH ALLOWS CORRECTLY FOR ERRORS IN THE ESTIMATES OF THE REGRESSION LINE AND IN THE RESPONSES. The confidence limits around the regression line are equally spaced in a vertical direction from each point on the line. M_L and M_U are asymmetrically placed about M. When the value of g is very small the limits in b and in c are practically identical.

the figure. The "expanded" variance [2] and the confidence limits (M_L and M_U) for M were calculated (Figure 1) according to the rigorous formula (4) which incorporates terms for the error in the regression line and for the difference between the mean response to unknown ($\bar{y}_u$) and to the reference standard ($\bar{y}_s$). A comparison between approximate formulas for the confidence limits and the rigorous formula is made schematically in Figure 2.

Log dose-response curves were analyzed by the usual regression methods (5). The "paired-data" t test was used to compare correlated slopes such as those in Table II. "Heterogeneity" of slopes in different assays or of M values from different assays for the same unknown was tested by the method of Cochran (6, 7), which is illustrated, for M values, by Bliss (3, 8).

Results reported as not significant had a probability (p) > 0.05 under the appropriate null hypothesis.

Log dose-response curves

A study of the response to graded doses of insulin in the range 31 to 1,000 μU per ml was made in a balanced segment experiment (Table I) performed on 5 consecutive assay days (Figure 3). A highly significant ($p < 0.001$) linear regression on log dose was observed on each day, and the five slopes did not vary significantly from each other. Curvature was not significant on any day, or in the pooled results. Comparable results were obtained in eight subsequent similar assays (Table II) except that in two instances significant deviations around the straight line were present.

Because many of these results showed an apparent, though nonsignificant, flattening at the upper part of the curve, the regressions were recalculated for the dose range 31 to 500 μU per ml. This calculation resulted in an increased slope and a slightly lower index of precision ($\lambda = s/b$) in 11 of the 13 cases (Table II). The difference in the two sets of slopes was highly significant ($p < 0.001$). Deviations from the regression line were now significant in only one assay. The combined findings, therefore, suggested that the 1,000 μU per ml concentration might be beyond the linear portion of the dose-response curve although individual assays with three rats were not, in general, sufficiently powerful to detect this phenomenon.

Routine assays

In the period March 1958 through March 1960, several modifications were introduced in the design of the assays and in the doses of standard insulin used. The assays

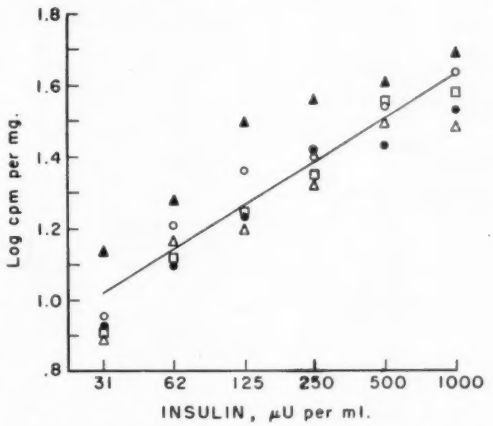

FIG. 3. DOSE-RESPONSE CURVES OBTAINED ON FIVE DIFFERENT DAYS. Each point shows the mean log counts per minute per milligram of tissue from three rats; the line is the calculated mean log dose-response curve.

[2] This "expanded" variance is identical with Bliss's (3) use of $L^2/4t^2$ where L is the confidence interval for an estimate.

TABLE II

Summary statistics showing response to log dose of standard insulin *

Assay date	Residual standard deviation (s)	Response to 6 doses (range 31–1,000 μU) b	Response to 6 doses (range 31–1,000 μU) λ	Response to 5 doses (range 31–500 μU) b	Response to 5 doses (range 31–500 μU) λ
5 Day experiment					
4/6/59	0.083	0.405	0.205	0.399	0.208
4/7/59	0.095	0.450	0.211	0.503	0.189
4/8/59	0.081	0.415	0.195	0.448	0.181
4/9/59	0.149	0.388	0.384	0.455	0.327
4/14/59	0.094	0.357	0.263	0.404	0.233
Subsequent assays					
5/13/59	0.075	0.415	0.181	0.469	0.160
8/5/59	0.115	0.377	0.305	0.430	0.267
8/27/59	0.094	0.331	0.284	0.325	0.289
9/25/59	0.071	0.502†	0.141	0.581†	0.122
9/28/59	0.109	0.425	0.256	0.486	0.224
9/30/59	0.133	0.477	0.279	0.518	0.257
10/2/59	0.145	0.424	0.342	0.492	0.295
10/5/59	0.062	0.375†	0.165	0.448	0.138
Mean‡	0.100	0.406	0.264	0.448	0.231
Standard deviation‡	0.028	0.041	0.063	0.053	0.057

* Each line is based on responses, in log cpm per mg, from three rats.
† Significant deviations from linearity ($p < 0.05$).
‡ In the calculations for mean and standard deviation, the assays with significant curvature were omitted.

were divided, according to these modifications, into the four series shown in Table III and Figure 4. To study the characteristics of the method under routine laboratory conditions, a "routine assay" was defined as one which: *a*) was intended for the study of the response to substances other than solutions of standard insulin; *b*) conformed to the design of its period; and *c*) included three responses to each of at least two dose levels of standard insulin.

TABLE III

Summary of 147 routine assays for which the log counts per minute were analyzed

Series	I	II	III	IV
Dates	3/5/58–4/3/59	4/13/59–5/27/59	6/10/59–10/19/59	11/6/59–3/31/60
Doses of insulin standard (μU/ml)	62–1,000	62–1,000	31–1,000	31–500
Balanced segment design	No	Yes	Yes	Yes
No. of assays included	45	16	31	55
Mean ± observed standard deviation:				
s	0.085 ± 0.031	0.103 ± 0.026	0.106 ± 0.032	0.102 ± 0.039
b	0.262 ± 0.071*	0.318 ± 0.090	0.356 ± 0.082	0.446 ± 0.068
λ	0.340 ± 0.143*	0.349 ± 0.117	0.313 ± 0.126	0.232 ± 0.092
Number of assays with significant† rat variation	39	8	20	31
Proximal segment giving lowest response:				
No. of assays		8	27	49
No. of assays in which difference was significant†		1	9	23

* Does not include 5 assays with nonsignificant slope on standard.
† $p < 0.05$.

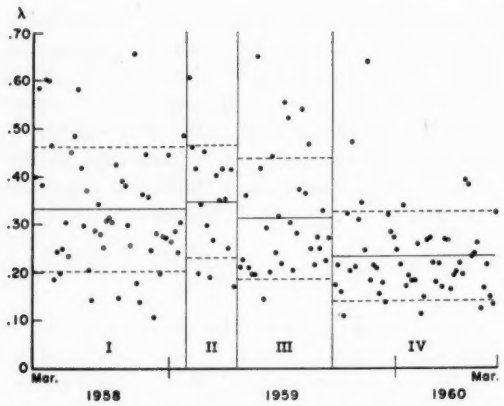

FIG. 4. VALUES OF λ OBTAINED IN ROUTINE ASSAYS WITH SIGNIFICANT SLOPES PERFORMED DURING THE PERIOD MARCH, 1958 THROUGH MARCH, 1960. Assays analyzed by $\sqrt{cpm}$ have been included and their λ values shown by open circles. In each part of the figure, the solid line represents the mean λ and the broken lines indicate one standard deviation on each side of the mean. Roman numerals refer to series identified in Table III.

A statistical analysis of the log counts per minute per milligram of tissue was made of all 30 routine assays performed in the three month period, May to July, 1959, and all of the 55 routine assays performed in the five month period, November 1959 through March 1960. The other routine assays summarized in Table III were chosen for analysis for a variety of reasons, mainly that of deriving estimates of ILA for certain human sera and pancreatic extracts.

Five of the assays analyzed, all in Series I, were nonvalid because the responses to standard insulin failed to show a significant slope. Where the slope was significant, the values of b and of λ in the assays of the first three series were similar, but an appreciable and statistically significant ($p < 0.01$) change may be observed in the statistics from Series IV assays (Figure 4).

A detailed tabulation of results in consecutive assays from Series III and IV is shown in Table IV. In each part of the table, the variation in the mean log counts per minute observed with the low dose of insulin was comparable to the variation in the mean log counts per minute with the high dose. This finding stands in contrast to the unequal variation (heteroscedasticity) that is observed in the counts per minute themselves at different levels of response (1).

The responses to the 31 μU per ml concentration of standard insulin were considerably lower ($p < 0.001$) in the Series IV assays in Table IV than in the Series III assays in the same table. Subsequent assays of series IV have maintained approximately the same level of response. It is possible that the relatively high slopes obtained in Series IV are due primarily to this phenomenon. Such differences between large groups of animals may represent seasonal or other differences in the metabolic state of the animals used, differences occurring despite the precautions employed in the effort to prevent them.

TABLE IV

Data from two groups of consecutive routine assays *

Assay date	Mean response to standard insulin: Low dose	High dose	s†	b	λ
A. 18 consecutive assays from Series III					
Standard insulin (μU/ml)	31	1000			
6/10/59	0.723	1.217	0.069	0.327	0.211
6/15	0.823	1.410	0.088	0.389	0.226
6/17	0.933	1.503	0.136	0.378	0.360
6/18	0.907	1.520	0.085	0.406	0.209
6/24	1.057	1.743	0.089	0.455	0.196
6/26	0.987	1.747	0.099	0.504	0.196
7/1	1.060	1.494	0.186	0.287	0.648
7/6	0.963	1.380	0.115	0.276	0.417
7/7	0.940	1.410	0.045	0.312	0.144
7/9	0.750	1.303	0.107	0.367	0.292
7/10	1.035	1.630	0.079	0.395	0.200
7/14	1.057	1.513	0.134	0.303	0.442
7/15	1.197	1.687	0.078	0.325	0.240
7/16	0.983	1.563	0.122	0.384	0.318
7/23	1.000	1.687	0.099	0.455	0.218
7/27	1.103	1.550	0.163	0.296	0.551
7/29	1.060	1.576	0.177	0.341	0.519
7/30	1.023	1.513	0.098	0.325	0.302
Mean	0.978	1.525	0.109	0.362	0.316
Standard deviation	0.120	0.145	0.037	0.064	0.144
B. 21 consecutive assays from Series IV					
Standard insulin (μU/ml)	31	500			
11/6/59	0.747	1.317	0.084	0.472	0.177
11/10	0.823	1.263	0.077	0.358	0.216
11/17	0.477	1.197	0.095	0.597	0.159
11/18	0.853	1.450	0.055	0.494	0.111
11/19	0.840	1.353	0.137	0.425	0.322
11/20	0.717	1.220	0.084	0.414	0.202
11/27	0.813	1.433	0.246	0.522	0.472
11/30	0.723	1.167	0.077	0.369	0.210
12/2	0.763	1.257	0.126	0.408	0.310
12/9	0.883	1.350	0.134	0.385	0.348
12/10	0.963	1.447	0.097	0.398	0.245
12/11	0.587	1.083	0.262	0.412	0.637
12/14	0.540	1.097	0.084	0.462	0.182
12/16	0.503	1.123	0.111	0.513	0.216
12/17	0.633	1.110	0.082	0.395	0.209
12/18	0.548	1.150	0.077	0.500	0.155
12/21	0.787	1.260	0.070	0.393	0.178
12/22	0.447	0.933	0.055	0.404	0.136
12/28	0.567	1.030	0.122	0.384	0.318
12/29	0.720	1.023	0.071	0.252	0.280
12/30	0.667	1.113	0.100	0.370	0.270
Mean	0.695	1.208	0.107	0.425	0.255
Standard deviation	0.146	0.146	0.054	0.074	0.122

* Each line is based on responses, in cpm per mg, from three rats.

† d.f = 8 except in assays 6/18 and 7/27 where d.f. = 7.

TABLE V

Minimum 95 per cent confidence ratio for potency estimates in two standard insulin dose ranges for an assay with six responses to standard insulin and three responses to a single level of an unknown serum

λ	Approximate ratio which ignores g	Accurate estimates of minimum ratio in two standard insulin dose ranges	
		31–1,000 μU	31–500 μU or 62–1,000 μU
0.10	2.1	2.1	2.1
0.15	3.1	3.2	3.2
0.20	4.5	4.7	4.9
0.25	6.5	7.2	7.7
0.30	9.5	11.3	12.8
0.35	13.8	18.6	23.0
0.40	20.2	32.0	46.5
0.45	29.3	59.4	114.7
0.50	42.8	122.0	401.0
0.55	62.1	292.0	3,072.0

Effectiveness of assay design. Variation between the tissues of different rats was significant and often very large in 67 per cent of the routine assays reported here. In these cases, the procedure of making all comparisons within rats reduced the residual error term by a factor of 2 to 10 (9).

The difference between segments was less marked and less consistent. The proximal segments gave lower responses in 5 of the 13 standard curves in Table II; the difference was significant at $p < 0.05$ in 2 of these. Similarly, the proximal segments gave lower values in 84 out of the 102 routine assays (significantly lower in 33) in Series II through IV. The overall finding of lower values in 84 out of 102 assays is highly significant ($p < 0.001$) by a χ^2 test (5).

Maximum precision of estimates. A useful measure of the precision of an assay is the "95 per cent confidence ratio" (10–12) defined as the ratio of the upper limit of the 95 per cent confidence interval of a potency estimate to the lower limit of the same interval. This ratio is equal to the antilogarithm ($\log^{-1}$) of $L = M_U - M_L$. Using the notation of Figure 1, the rigorous calculations yield:

$$L = 2t\lambda \sqrt{\frac{1}{1-g}} \sqrt{\frac{N_s+N_u}{N_sN_u} + \frac{(\bar{y}_u-\bar{y}_s)^2}{SSR\ (1-g)}}. \quad [1]$$

When $\bar{y}_u = \bar{y}_s$, Equation 1 reduces to the minimum L where

$$\min L = 2t\lambda \sqrt{\frac{1}{1-g}} \sqrt{\frac{N_s+N_u}{N_sN_u}}. \quad [2]$$

The antilogarithm of Equation 2 is the minimum confidence ratio attainable in a particular assay. The statistic g in Equations 1 and 2 varies with the error in the estimate of slope. When this error is sufficiently small to make $g < 0.05$, it may be omitted from the calculations (2, 13). Under these conditions Equation 2 reduces to the formula of Figure 2a, namely,

$$Q = 2t\lambda \sqrt{\frac{N_s+N_u}{N_sN_u}}. \quad [3]$$

The value of t in these formulas depends on the level of confidence chosen (95 per cent) and on the degrees of freedom (d.f.) for s^2. In the present design there are 8 d.f. giving $t = 2.306$. The value of $\frac{N_s+N_u}{N_sN_u}$ depends on the number of responses contributing to an individual potency estimate, and in the routine assay design it is equal to 0.5. If these constants are substituted in Equations 2 and 3, the formulas reduce, in this routine design, to:

$$\min L = 3.26\lambda \sqrt{\frac{1}{1-g}} \quad [4]$$

$$\text{and } Q = 3.26\lambda. \quad [5]$$

The statistic g is related to the index λ as is shown by the algebraic identities:

$$g = \frac{s^2t^2}{SSR} = \frac{s^2t^2}{b^2r\Sigma(x-\bar{x})^2} = \frac{\lambda^2t^2}{r\Sigma(x-\bar{x})^2}. \quad [6]$$

In the routine assay design, $t^2/r\Sigma(x-\bar{x})^2$ depends only on the doses of standard insulin given. For the dose ranges used in these assays, the following values may be calculated:

Standard insulin per ml	$3\Sigma(x-\bar{x})^2$	g
31 μU—1,000 μU	3.414	$1.558\ \lambda^2$
31 μU— 500 μU or 62 μU—1,000 μU	2.187	$2.434\ \lambda^2$

The minimum L to be expected for values of λ ranging from 0.10 to 0.55 was calculated by inserting the appropriate values of 1/(1-g) into Equation 4. The corresponding minimum confidence ratios are shown in Table V where they may be compared with the approximations given by Equation 5. It is clear that in small assays of this type, use of the approximate formula will lead to appreciable underestimation of the minimum error of potency estimates unless λ is < 0.20. The small number of responses per assay produced the phe-

TABLE VI

Results of repeated assays of same sera

Subject	Date when sample was obtained	Assay date	Potency estimate of ILA (μU/ml)	Log potency (M)	Expanded variance predicted for M	Observed variance	p Value in test for heterogeneity
L.G.	9/8/59	9/10	582	2.765	0.1005	0.0420	>0.30
		9/14	335	2.525	0.1756		
		9/16	597	2.776	0.0400		
		10/14	224	2.350	0.0276		
J.K.	9/15/59	9/17	336	2.526	0.0259	0.0147	>0.50
		10/6	212	2.326	0.0342		
		10/14	351	2.545	0.0289		
G.C.	11/–/58	12/1	26	1.407	0.0134	0.0535	>0.10
		12/15	54	1.734	0.0269		
	8/20/59	8/28	64	1.804	0.0240	0.0892	>0.50
		9/2	32	1.511	0.0600		
		9/3	25	1.391	0.1399		
		9/8	21	1.318	0.5227		
		9/21	31	1.490	0.0581		

nomenon, notable in Table V, of a rapid expansion in the minimum confidence ratio with an increase in the value of λ.

The confidence ratios may be reduced by increasing the number of responses per assay. Depending on the design (*2*) such an increase could: reduce the value of t by its effect on the d.f.; reduce the magnitude of $(N_s + N_u)/N_sN_u$; increase the value of $r\Sigma(x-\bar{x})^2$; and reduce the size of g. For example, if six rats were used in the routine assay of four unknowns with no other change in the design, the 95 per cent minimum confidence ratios for an assay where $\lambda = 0.30$ would be 6.6 for the approximate ratio, and 7.0 and 7.2 for the accurate estimates in the two dose ranges shown in Table V, a decrease of approximately 40 per cent in the accurate ratios.

Interassay variation. The foregoing section refers to the reproducibility of assay results as estimated from the statistics within individual assays. Accumulated experience, however, permits direct comparisons of results in different assays and, therefore, comparisons between predicted and observed reproducibility (14).

The slopes of the different assays within Series I and Series IV did not vary significantly more than expected for estimates based on two responses per rat. In the other two series the observed variance between slopes was approximately double the predicted variance ($p < 0.05$). This common phenomenon is of no practical significance when standard slopes are run in each assay. The acid test of interassay variation is the reproducibility of M values obtained in different assays of the same unknown. Estimates from two to five independent assays of four different sera are shown in Table VI together with the "expanded" variance of M calculated in each assay, and with the variance calculated from the observed M values. It is clear both from the p values in the test for heterogeneity and from the comparison of observed with predicted variances that the interassay variation among these M values was not greater than predicted.

RESULTS

Tests of "normal" human sera

Technically valid estimates [3] of ILA were available for 50 undiluted serum samples obtained from 30 fasting, apparently normal human subjects. The 95 per cent confidence ratios were > 100 for 12 of these estimates obtained in a total of eight different assays. In two assays there were fewer than 15 usable responses; in the other instances large "expanded" standard errors of M were produced by a combination of λ values > 0.40 with mean responses for the unknowns that were at one of the extremes of the standard curve.

The remaining 38 estimates of ILA involving 23 subjects are shown in Table VII as microunits of insulin per milliliter of serum, together with

[3] These were derived from assays with significant slopes.

TABLE VII

Estimates of ILA of 38 undiluted serum samples from 23 fasting normal individuals *

Subject	Sex	Age	ILA in μU/ml serum† Estimate	95% Confidence limits
P.B.	M	37	510	200–1,600
H.B.	M	24	35	7– 92
R.B.	M	26	140	38– 390
Z.C.	M	38	310	94–1,200
A.F.	M	25	300	130– 790
R.H.	M	29	440	150–1,600
S.M.	M	25	840	380–2,300
G.Z.	M	33	440	170–1,600
D.C.	F	24	810	260–4,210
U.D.	F	22	130	28– 430
A.E.	F	23	360	170– 830
V.S.	F	39	350	180– 690
G.C.	M	33	33‡	21– 53
			43‡	26– 72
Y.D.	M	33	190	73– 460
			300	100– 920
			100	24– 290
			510	230–1,300
			130	10– 570
P.D.	M	?	44	32– 110
			180	100– 300
B.J.	M	28	89	15– 270
			270	64–1,400
J.K.	M	34	310	110–1,000
			300‡	190– 480
B.L.	M	30	390	130–1,600
			35	6– 94
H.M.	M	29	100	22– 320
			290	170– 490
U.M.	M	27	360	150– 970
			450	210–1,100
A.R.	M	35	89	40– 170
			300	60–1,800
			940	220–1,200
F.W.	M	31	150	37– 460
			230	48–1,100
L.G.	F	28	150	78– 440
			360‡	210– 610

* Repeated samples from an individual were taken on separate occasions.

† Rounded to two significant digits.

‡ Derived from weighted means of multiple estimates in Table VI.

their 95 per cent confidence limits. The estimates ranged between 33 and 940 μU per ml.

ILA of serum following infusions of glucose and of mannose [4]

Samples of blood were collected from nondiabetic fasting subjects in apparent good health, just before an intravenous infusion of either glucose or mannose (0.5 g per kg body weight in a 50 per cent solution given over 3 minutes) and also 10, 20 and 60 minutes after the infusion. All four samples from any one subject were tested together in one assay.

Serum glucose levels after the infusion of glucose showed the expected pattern, the mean values for the subjects in Figure 5 being 75, 275, 212 and 82 mg per 100 ml in the four time periods. Following mannose infusion, glucose values varied randomly within normal limits, while the mean serum mannose dropped from a peak of 216 mg per 100 ml at 10 minutes to 90 mg per 100 ml at 1 hour.

The sera from seven subjects in the glucose experiments were tested in eight assays, two of which failed to show a significant slope in the response to standard insulin. Potency estimates could be calculated for four subjects from one assay each; for a fifth subject (G.C.) estimates of ILA were derived from weighted mean M's from two assays (15).

Five subjects were given mannose infusions. In the assays of their sera, mannose was added to all the media in a concentration equal to the mean mannose content of the sera tested (200 to 230 mg per 100 ml). The usual response to standard insulin, with significant slopes, was seen in all five assays.[5] Estimates of ILA were calculated from these assays in the usual way, for comparative purposes, although they were not included in Table VII.

Twenty minutes after the infusion of glucose, the ILA of the serum of each subject was in-

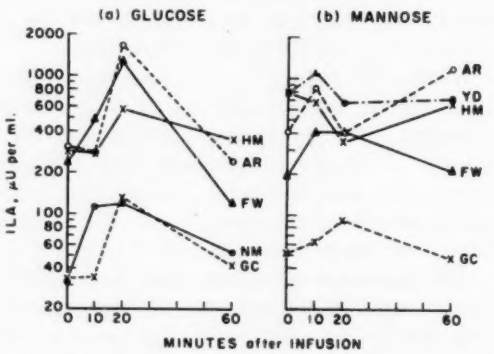

FIG. 5. ESTIMATES OF ILA IN HUMAN SERA PLOTTED ON A LOGARITHMIC SCALE: a. FOLLOWING INFUSION OF GLUCOSE; b. FOLLOWING INFUSION OF MANNOSE.

[4] The mannose infusions were carried out in collaboration with Drs. Francis C. Wood, Jr. and George F. Cahill, Jr.

[5] Not included in the assay summary because of the added mannose.

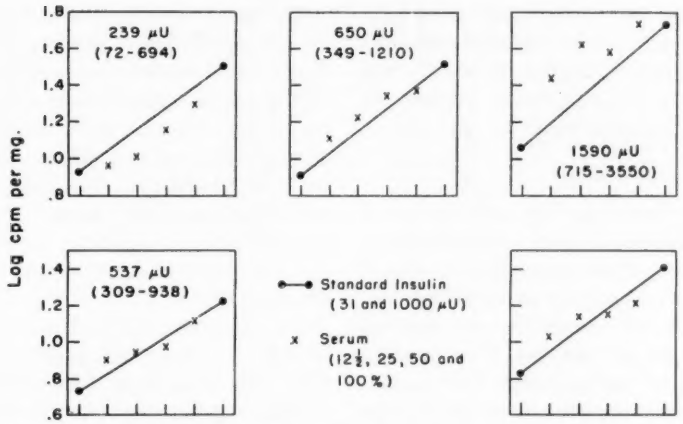

FIG. 6. THE RESPONSE OF ADIPOSE TISSUE TO SERIAL DILUTIONS OF HUMAN SERUM AND TO STANDARD INSULIN. The dose of insulin or serum is shown on the abscissa. The estimates of ILA with 95 per cent confidence limits in microunits per milliliter, as calculated from all the data, are shown where there was no significant nonparallelism.

creased (Figure 5) by a factor which varied from 100 to 440 per cent. The increase was significant (at $p < 0.05$) in all cases but one (N. M.). In contrast, after the infusion of mannose the ILA varied in a random fashion (Figure 5) and none of the differences reached statistical significance.

To form an approximate estimate of the power of these assays to detect changes in ILA, the statistics in each assay were used to calculate the smallest difference between two means of three responses each that would be significant at $p < 0.05$ by a one-sided t test. For each "least significant difference," the corresponding increase in potency was calculated, and ranged from 40 to 330 per cent (i.e., a potency 1.4 to 4.3 times the pre-infusion value). A least significant difference was also calculated for samples tested in separate assays (15). The calculations, which were based on the *minimum* "expanded" variance of an M from each assay, appreciably underestimated the differences which would actually have been required for statistical significance. Even these underestimates indicated that, to be detected, samples compared in different assays would have to show at least twice as much change as could be detected within an assay.

The response to diluted sera

The effect of diluting human sera was tested in five assays. In each instance a sample of serum, taken from a fasting normal volunteer, was tested full strength and also after dilution with buffer to 50, 25 and 12.5 per cent strength. The results are shown in Figure 6. The slope of the response to standard insulin was highly significant ($p < 0.01$) in each assay. In one assay, there was significant ($p < 0.05$) nonparallelism between the slope on standard insulin and on serum. In the other four assays estimates of ILA and of 95 per cent confidence limits (Figure 6) were calculated from all the data with a "combined" estimate of the slope based on the responses to both the serum and the standard insulin (2, 8).

The values for λ, calculated from the combined slope, were 0.36, 0.22, 0.22 and 0.23, in the order shown in Figure 6. The 95 per cent confidence ratios, calculated according to Equation 1, were: 9.6, 3.5, 5.0 and 3.0. These ratios were smaller than the minimal ratios (Table V) corresponding to similar λ values, both because all 18 responses in an assay contributed to the estimate of slope (thus diminishing the value of g), and because 12 responses for a test serum were available for each estimate of potency.

On the other hand, a question must be raised about the effect of dilution on the serum. Although there was no significant nonparallelism in four of the assays, the slope of the response to serum was flatter than the standard slope in each instance. This result in the five cases combined

was significant ($p < 0.05$). Estimates of the relative potency of the 12.5 per cent dilution computed for the five examples in Figure 6 were, in the order shown, 110, 170, 170, 170 and 270 per cent of the potency of the same serum before dilution.

DISCUSSION

This procedure meets the first major requirement for a quantitative biological assay method by exhibiting a relatively reproducible linear response to the logarithm of increasing doses of standard insulin. The results of experiments with multiple doses of standard insulin have been confirmed by the observations that over a period of 25 months significant regressions occurred in all but 5 of the 147 routine assays analyzed. The suggestion that the linear portion of the dose-response curve lies below the concentration 1,000 μU per ml, has been supported by the finding that the assays in Series IV (31 to 500 μU of insulin) had significantly steeper slopes and better λ values than the earlier series. The further observation that the variation in the response to high and low doses was of similar magnitude shows that the log counts per minute were a satisfactory response metameter for these assays.

Although potency estimates derived from these assays have a large error, the precision can be increased not only by increasing the size of individual assays, but also (Table VII) by reassaying an unknown on several days. The evidence available to date regarding the reproducibility of independent estimates of the potency of a sample of serum from assay to assay does not indicate the presence of important interassay error. The precision of the method compares favorably with that of other methods used to measure ILA in blood. For the rat diaphragm method, reported values (16, 17) of λ have been in the range 0.20 to 0.40. Willebrands, Groen and van der Geld (12, 18) have estimated a 95 per cent confidence ratio of 9.0 in assays with five responses each at single levels of standard insulin and of diluted serum. Since their calculations omit both the term $1/(1\text{-}g)$ and the term for $\bar{y}_u - \bar{y}_s$ in Equation 1, this estimate is comparable to the approximate minimum ratio calculated from Equation 3. From the data on the standard response presented (18) by these authors it may be calculated that the value of λ was approximately 0.50 to 0.60.

The use of epididymal adipose tissue offers the important advantage that all estimates for unknowns can be based on a response to two or more dose levels of standard insulin obtained from tissues of the same rats at the same time. It is now generally accepted that estimates made in this way are more reliable than those calculated from an independent, previously obtained, estimate of standard slope. The importance of this feature is indicated by the variation observed between the slopes of assays in Series II and III.

The possibility of giving a number of different treatments to the tissue from each rat has several additional advantages. One set of responses to standard insulin provides the basis for potency estimates of several unknowns, thereby producing more efficient utilization of animals and laboratory time. Moreover, this opportunity to compare several sera or other unknowns within an assay adds considerably to the power of the method. A difference between two samples tested in one assay is subject only to the experimental variation affecting the responses to the two samples. Two potency estimates from two separate assays are each subject to experimental variation affecting a mean response to the unknown and to standard insulin, and to variation affecting an estimate of slope. As a result, a "between-assay" comparison of two potency estimates is subject to variation affecting the responses both to the unknowns, and to two separate sets of standard insulin doses. With a given degree of precision per assay, therefore, and no interassay variation, within-assay comparisons can detect smaller differences than can between-assay comparisons.

The effect on ILA of diluting complex samples such as sera, has important practical implications for the use of this assay method, in addition to its physiological interest and its relevance to the fundamental validity of the assay method (2). Although in pharmacology nonparallel behavior of the responses to unknowns and to the standards suggests nonidentity, in complex biological samples other interpretations have to be considered. For example, the activity may be present in different chemical or physicochemical states (such as "bound" and "free") related to each other by equilibria sensitive to dilution. Still, unless serum does produce responses that are parallel to the reference standard, potency estimates derived from

diluted serum and even from undiluted serum must be interpreted with special caution.

The preliminary results reported here suggest that if dilution of serum does produce a relative increase in potency as shown by the response of epididymal adipose tissue, this effect is considerably less pronounced than the increase observed in assays utilizing the rat diaphragm. For example, from the data presented by Randle (19), it may be calculated that dilution of serum to 25 per cent altered ILA to 80, 320, 330, 400 and 660 per cent of that observed before dilution. Willebrands, van der Geld and Groen (18) reported estimates for sera diluted to 10 per cent which were 350 to 1,300 per cent of estimates made from more concentrated preparations of the same sera. In the light of the smaller discrepancies (110 to 270 per cent ILA in diluted sera) observed in this laboratory, further study is being given to the feasibility of estimating the total ILA of undiluted serum from assays performed on diluted serum. If this procedure should prove possible, the scope of the assay method would be appreciably increased.

The data on serum ILA which have been presented are intended to illustrate the possible applicability of the procedure to complex biological samples. This in no way implies that the activity measured in serum was indeed insulin. Studies related to the nature of the ILA of serum will be published after they have been completed, although it may be pointed out at this time that the response to glucose (and not to mannose) suggests an insulin component. Similarly, serum ILA measured by this procedure is higher in pancreatic and portal venous blood than it is in the general circulation (20), and is frequently elevated in serum obtained from patients with tumors of pancreatic islets with hypoglycemia (21). ILA in serum is also increased after the intravenous administration of insulin and recoveries of insulin added to serum are satisfactory.[6] A major portion of serum ILA is destroyed by incubation with reduced glutathione.[6] However, serum ILA does not appear to be reduced below the normal range in pancreatectomized dogs (22) or cats.[7] Accordingly, considerable work with isolation, extraction and characterization procedures will be required before a definitive statement on the nature of ILA in serum can be made.

[6] Steinke, J., Dagenais, Y. M., and Renold, A. E. Unpublished observations.

[7] Steinke, J., Lukens, F. D. W., and Renold, A. E. Unpublished observations.

SUMMARY AND CONCLUSIONS

Experiments and routine assays performed over a period of two years and involving over 500 rats indicate that it is feasible to measure minute concentrations of insulin (or of "insulin-like activity") by utilizing measurements of the $C^{14}O_2$ produced from glucose-1-C^{14} by epididymal adipose tissue of rats. The tissue response is recorded as the logarithm of the counts per minute of CO_2 produced per milligram of adipose tissue (wet weight). After incubation with standard insulin in concentrations of 31 to 500 μU per ml, the log counts per minute increase linearly with the log dose of insulin.

The utilization of epididymal adipose tissue permits assays to be so designed that simultaneous comparisons between standard insulin and several "unknowns" may be made while eliminating differences between rats and between the several segments of each epididymal fat pad. Six different combinations of substances and concentrations are tested in each experiment or assay. The design for a routine assay was presented together with the method of analyzing the results and of calculating accurate confidence limits.

Data from 147 routine assays were summarized. Significant slopes in the response to standard insulin were found in all 102 analyzed assays performed with the balanced segment design. The last series of 55 assays, in which standard insulin was given in concentrations of 31 and 500 μU per ml, had a mean index of precision (λ) of 0.23. With small assays, this value of λ still implies a rather large error, although the error may be decreased by modifying the design of an assay. The precision is at least comparable to that of other methods presently in use.

Within individual assays, each utilizing tissues from three rats, the method was sufficiently precise (or powerful) to detect an increase in serum insulin-like activity 20 minutes after the intravenous infusion of glucose.

Preliminary data suggest that potency estimates are reproducible from assay to assay, within the error of the method. Other preliminary experi-

ments suggest that, by this method, there may be less discrepancy in insulin-like activity following dilution of complex samples such as serum than has been observed in the rat diaphragm assay procedure.

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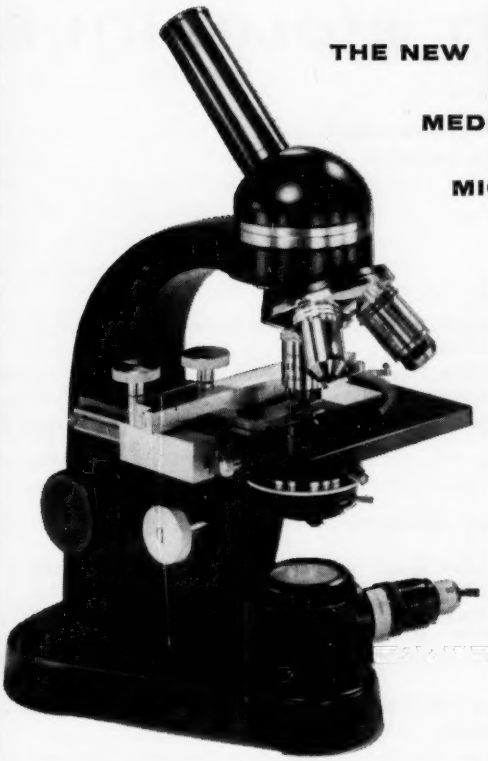

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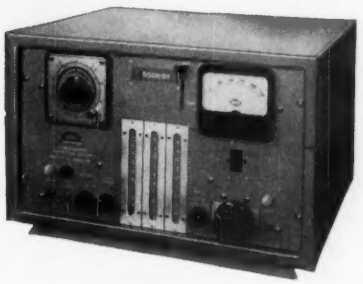

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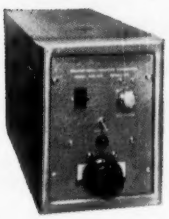

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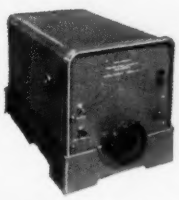

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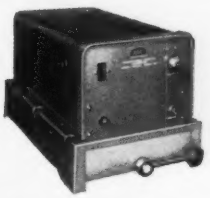

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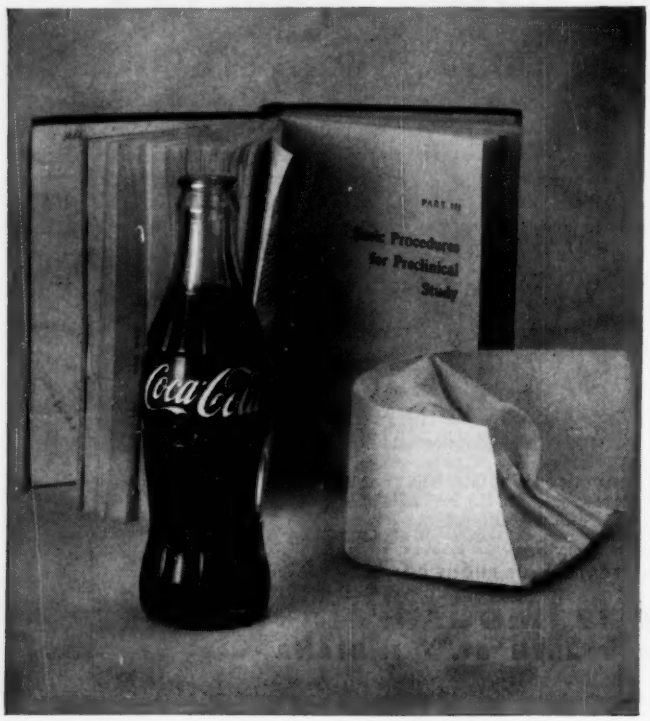

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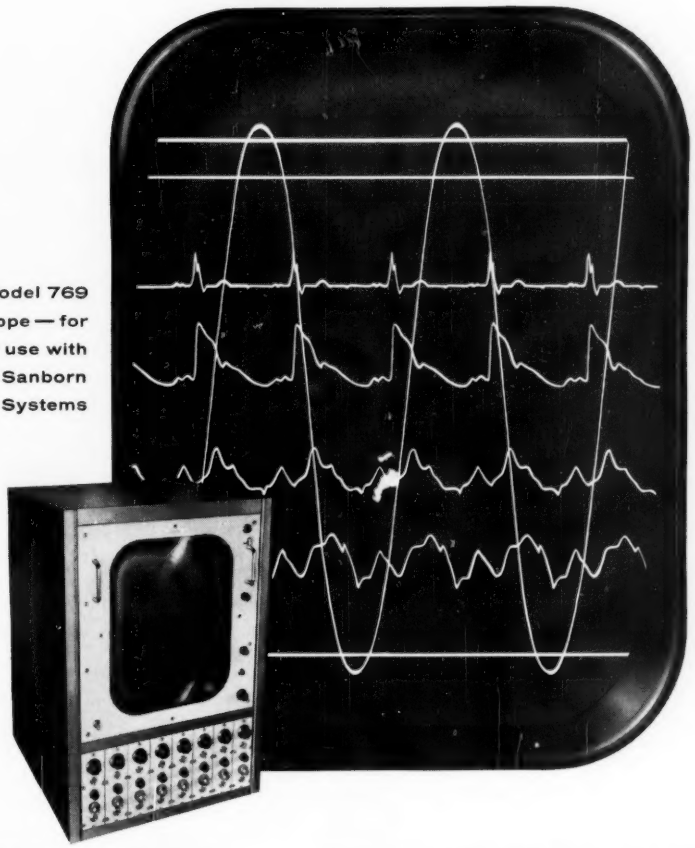

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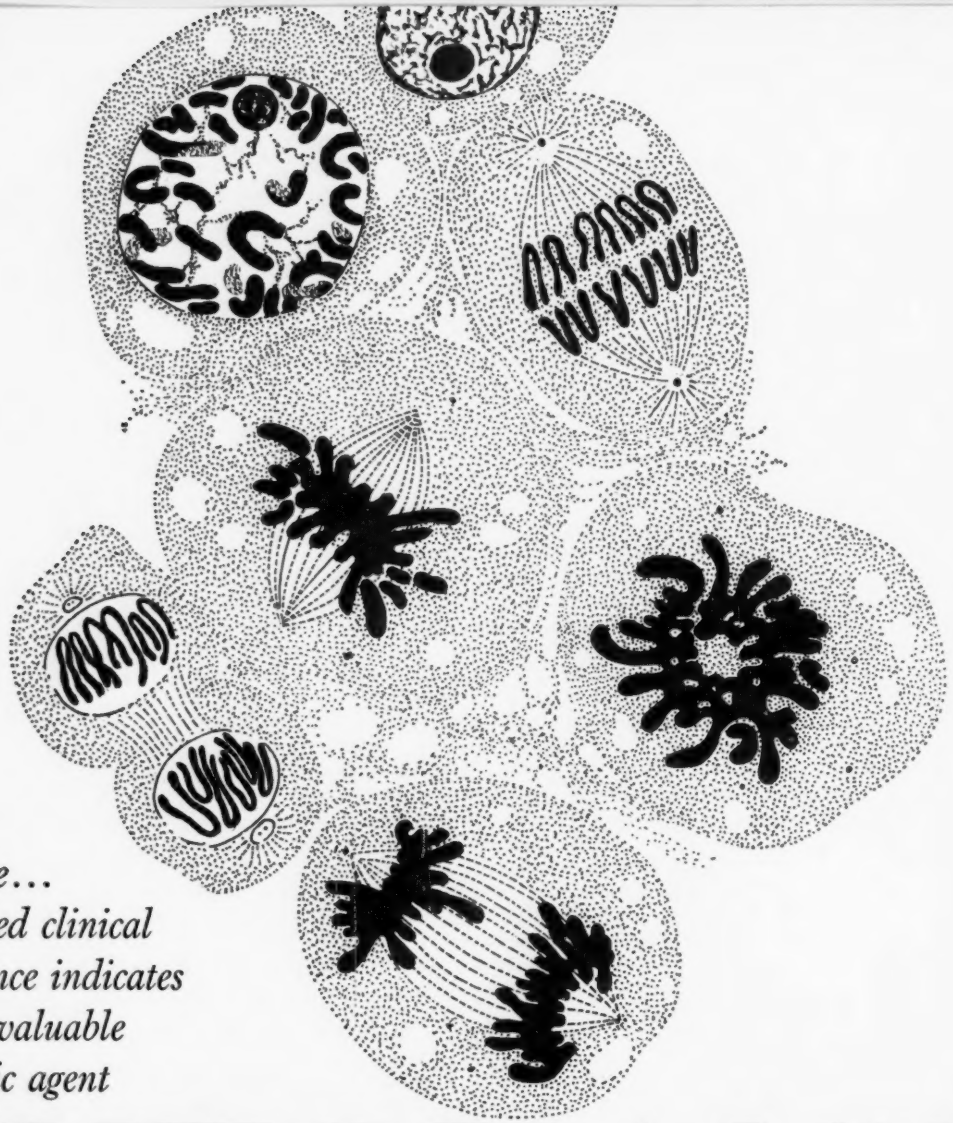

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*Papac, R.; Petrakis, N. L.; Amini, E. and Wood, D. A.: J.A.M.A. *172*:1387-1391 (March 26) 1960.

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